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| (54) Title: MODIFIED ANTIBODIES AND ANTIBODY FRAGMENTS WITH INCREASED DURATION OF ACTIVITY   |           |   |
| <p>(57) Abstract</p> <p>Modified antibodies with improved pharmacokinetic properties are disclosed. The antibodies bind to one or more human integrin receptors selected from the group consisting of GPIIb/IIIa, <math>\alpha_v\beta_3</math> and Mac-1, and comprise one to about six organic moieties which are covalently bonded to specific sites on the antibodies. The organic moieties can each independently be a hydrophilic polymeric group, a fatty acid group, a fatty acid ester group, a lipid group or a phospholipid group. Also disclosed is a method of treating a human with a disease or disorder in which cells expressing GPIIb/IIIa, <math>\alpha_v\beta_3</math> and/or Mac-1 play a pathophysiological role. The method comprises administering an effective amount of one or more antibodies of the invention to a human in need thereof.</p>         |           |   |

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## MODIFIED ANTIBODIES AND ANTIBODY FRAGMENTS WITH INCREASED DURATION OF ACTIVITY

### RELATED APPLICATION

5 This application is a continuation-in-part of U.S. serial number 09/185,151,  
filed November 3, 1998, the entire teachings of which are incorporated herein by  
reference.

### BACKGROUND OF THE INVENTION

10 Platelets play a key role in regulating hemostasis by binding to sites of injury  
and, upon activation, initiating the biochemical events which result in formation of  
a clot. However, under certain circumstances platelets can exacerbate  
pathophysiological conditions such as cardiovascular disease and tumor metastasis.  
For example, under normal circumstances platelets can adhere to a site of blood  
vessel injury through the interaction of receptors expressed on the plasma membrane  
15 of the platelet with the subendothelial layer, thereby activating the platelets.  
Platelets can be activated by numerous other stimuli including thrombin, adenosine  
diphosphate (ADP), contact with artificial surfaces and some immune complexes.  
Once activated, platelets expose the fibrinogen binding sites on the plasma  
membrane glycoprotein GPIIb/IIIa and platelet aggregation takes place via  
20 fibrinogen bridging. Aggregation of platelets can trigger degranulation of  
cytoplasmic vesicles, releasing proteins and chemical mediators into the local  
extracellular space, which can lead to further platelet adhesion, activation and  
aggregation to generate a clot which prevents the escape of blood cells from the  
vessel. However, under certain conditions platelet aggregation can occlude a blood  
25 vessel leading to ischemia and cellular necrosis. For example, platelet aggregation  
and clot formation at a ruptured atherosclerotic plaque in a coronary artery can lead  
to angina or acute myocardial infarction. Further, platelet aggregation and clot  
formation in blood vessels at sites of endothelial cell injury or denudation, as can be  
found following angioplasty, can lead to abrupt closure of the vessel necessitating  
30 emergency intervention, such as by-pass surgery.

The hemostatic and pathophysiological functions of platelets, which require adhesion and aggregation of the cells, are mediated by the interaction of platelet plasma membrane receptors with ligands. These receptors (e.g., GPIIb/IIIa) are members of the integrin family of receptors which generally bind to matrix proteins (e.g., collagen, fibronectin, fibrinogen, laminin, vitronectin and von Willebrand factor) and are involved in immune function and tumor metastasis in addition to platelet function. Thus, antagonists of certain integrins (e.g., GPIIb/IIIa) hold great promise as therapeutic agents.

Several antagonists of GPIIb/IIIa have been developed including snake venom proteins, small peptides, peptidomimetics, non-peptide compounds and antibodies (Cook, N.S., *et al.*, *Drugs of Future*, 19: 135-159 (1994); Cox, D., *et al.*, *Medicinal Research Reviews*, 14:195-228 (1994); Coller, B.S., *et al.*, *Thrombosis and Haemostasis*, 74(1) 302-308 (1995)). A fragment of a humanized chimeric antibody, c7E3 Fab (also known as "abciximab" or ReoPro®), that binds GPIIb/IIIa (expressed specifically on platelets),  $\alpha_v\beta_3$  (expressed on platelets, endothelial cells and perhaps smooth muscle cells (Felding-Habermann, B. and Cheresch, D.A., *Current Opinion in Cell Biology*, 5:864-868 (1993)), and Mac-1 (also referred to as CR3, CD11b/CD18,  $\alpha_M\beta_2$ , which is expressed on activated leukocytes, such as monocytes and granulocytes (Altieri, D.C. and Edgington, T.S., *Journal of Immunology* 141:2656-2660 (1988)) has been used to prevent the abrupt closure of coronary arteries and to diminish ischemic complications following a coronary artery intervention procedure. However, therapy to mitigate or prevent certain other pathological events can require frequent administration of a therapeutic agent over an extended period of time. The efficacy of this type of therapeutic regimen can be unsatisfactory due to patient compliance issues. Additionally, the frequent administration of therapeutic agents (e.g., proteins or peptides) can elicit the production of antibodies that bind the agent. Thus, the patient can develop immune complex-mediated resistance and/or allergy to a particular therapeutic agent.

There is a continuing need for an integrin receptor antagonist with an extended duration of action and, preferably, low immunogenicity.

#### SUMMARY OF THE INVENTION

The invention relates to modified therapeutic antibodies. The modification  
5 serves to improve the pharmacokinetic properties (e.g., increased *in vivo* serum half-life) without significantly affecting the antigen-binding properties (e.g., affinity) of the antibodies.

The invention is particularly related to antibodies which are modified by the covalent attachment of an organic moiety. The modified antibodies of the invention  
10 bind to one or more of the integrin receptors selected from the group consisting of GPIIb/IIIa,  $\alpha_v\beta_3$  and Mac-1. In particular embodiments, the invention provides modified antigen-binding fragments of antibodies (e.g., Fv, Fab, Fab', F(ab')<sub>2</sub>). In a preferred embodiment, the modified antibodies bind to GPIIb/IIIa and/or  $\alpha_v\beta_3$  and antagonize the binding of fibrinogen to GPIIb/IIIa and platelet aggregation. In  
15 additional embodiments, the binding of the modified antibody of the invention to GPIIb/IIIa and/or  $\alpha_v\beta_3$  is competitively inhibited by 7E3. Preferably the modified antibody of the invention is 7E3 or a humanized 7E3. A preferred humanized 7E3 is c7E3.

The organic moiety can be a hydrophilic polymeric group, fatty acid group, a  
20 fatty acid ester group, a lipid group or a phospholipid group. In particular embodiments, the hydrophilic polymeric group can have a molecular weight of about 800 to about 120,000 Daltons and can be a polyalkane glycol (e.g., polyethylene glycol (PEG), polypropylene glycol (PPG)), carbohydrate polymer, amino acid polymer or polyvinyl pyrrolidone, and the fatty acid or fatty acid ester group can comprise from  
25 about eight to about forty carbon atoms.

The modified antibodies of the invention comprise one to about six organic moieties which are covalently bonded to a carboxyl-terminus of the antibody and/or covalently bonded to the sulfur atom of a cysteinyl residue of the antibody. Thus, the

invention provides antibodies comprising site-specific modifications. For example, a modified Fab of an IgG can comprise a PEG moiety, which is bonded to the carboxyl-terminus of the heavy chain.

5 In a particular embodiment, a modified c7E3 Fab' comprises two PEG moieties which are each bonded to the sulfur atom of one of the cysteinyl residues that are contained within the hinge region of the heavy chain (the cysteine residues in the hinge region which form inter-chain disulfide bonds in the corresponding IgG or F(ab')<sub>2</sub>).

In another particular embodiment, a modified c7E3 Fab generated through the action of achromopeptidase, comprises one PEG moiety which is bonded to the  
10 carboxyl-terminus of the heavy chain.

The invention also provides a method of treating or preventing diseases and/or disorders in which cells expressing GPIIb/IIIa,  $\alpha_v\beta_3$  and/or Mac-1 (e.g., platelets, endothelial cells, leukocytes) play a pathophysiological role. Such diseases and disorders include, for example, thromboembolic disorders including cardiovascular  
15 disease (e.g., atherosclerosis, angina, coronary artery disease), stroke, ischemia, and tumor vascularization, tumor metastasis and inflammatory conditions. The method comprises administering an effective amount of a modified antibody of the invention to a human in need thereof. The method can comprise the administration of an antibody of the invention alone or in combination with an effective amount of one or more  
20 additional active agents.

In another embodiment, the invention provides a method of inhibiting platelet-mediated metastasis in a human comprising administering an effective amount of a modified antibody of the invention to the human.

In additional embodiments, the invention provides a method of inhibiting a  
25 process (i.e., one or more) mediated through the binding of a ligand to GPIIb/IIIa,  $\alpha_v\beta_3$  or Mac-1 in a human comprising administering an effective amount of a modified antibody of the invention to a human in need thereof. GPIIb/IIIa is expressed specifically on platelets and binds several ligands (e.g., fibrinogen, fibronectin, von Willebrand factor, thrombospondin, vitronectin). The  $\alpha_v\beta_3$  vitronectin receptor is

expressed on cells such as endothelial cells and vascular smooth muscle cells, and to a lesser extent, on platelets, and mediates adhesion to a variety of extracellular matrix proteins (e.g., vitronectin, fibronectin, von Willebrand Factor, fibrinogen, osteopontin, thrombospondin, collagen, perlecan). Mac-1 is expressed on activated leukocytes, such as monocytes and granulocytes, and binds a variety of ligands (e.g., C3bi, Factor X, fibrinogen).

The present invention further relates to a modified antibody or fragment as described herein for use in therapy (including prophylaxis) or diagnosis, and to the use of such a modified antibody or fragment for the manufacture of a medicament for the treatment of a particular disease or condition as described herein (e.g., a thromboembolic disorder, a disease or disorder in which cells expressing GPIIb/IIIa and/or  $\alpha_v\beta_3$  play a pathophysiological role).

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing dose dependent inhibition of ADP-induced (15  $\mu$ M ADP) platelet aggregation (234,000/ $\mu$ L) by c7E3 Fab and c7E3 Fab'(PEG<sub>5000</sub>)<sub>2</sub>.

Figure 2 is a graph showing dose dependent inhibition of ADP-induced (20  $\mu$ M ADP) platelet aggregation (250,000/ $\mu$ L) by c7E3 Fab and c7E3 Fab'(PEG<sub>20,000</sub>)<sub>2</sub>.

Figure 3 is a graph showing that <sup>125</sup>I-c7E3 Fab competitively inhibits binding of c7E3 Fab and c7E3 Fab'(PEG<sub>20,000</sub>)<sub>2</sub> to GPIIb/IIIa on GPIIb/IIIa-coated plates.

Figure 4 is a graph showing that <sup>125</sup>I-c7E3 Fab competitively inhibits binding of c7E3 Fab and c7E3 Fab'(PEG<sub>20,000</sub>)<sub>2</sub> to  $\alpha_v\beta_3$  on  $\alpha_v\beta_3$ -coated plates.

Figure 5 is a graph showing that <sup>125</sup>I-c7E3 Fab competitively inhibits binding of c7E3 Fab and c7E3 Fab'(PEG<sub>5000</sub>)<sub>2</sub> to  $\alpha_v\beta_3$  on  $\alpha_v\beta_3$ -coated plates.

Figure 6 is a graph showing that <sup>125</sup>I-c7E3 Fab competitively inhibits binding of c7E3 Fab and c7E3 Fab'(PEG<sub>5000</sub>)<sub>2</sub> to GPIIb/IIIa on GPIIb/IIIa-coated plates.

Figure 7 is a graph showing that <sup>125</sup>I-c7E3 Fab competitively inhibits binding of c7E3 Fab and c7E3 Fab-PEG<sub>5000</sub> to GPIIb/IIIa on GPIIb/IIIa-coated plates.

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Figure 8 is a graph showing that  $^{125}\text{I}$ -c7E3 Fab competitively inhibits binding of c7E3 Fab and c7E3 Fab-PEG<sub>5000</sub> to  $\alpha_v\beta_3$  on  $\alpha_v\beta_3$ -coated plates.

Figure 9 is a graph showing the rate of clearance of c7E3 Fab'(PEG<sub>5000</sub>)<sub>2</sub> and c7E3 Fab'(NEM)<sub>2</sub> from the serum of mice.

5 Figure 10 is a graph showing the rate of clearance of c7E3 Fab'-(PEG<sub>20,000</sub>)<sub>2</sub> from the serum of mice.

Figure 11 is a graph showing the rate of clearance of c7E3 Fab'(PEG<sub>5000</sub>)<sub>2</sub> and c7E3 Fab from the serum of rats.

Figure 12 is a bar graph showing the titre of specific IgG antibody  
10 (anti-c7E3 Fab) produced by mice immunized with c7E3 Fab or c7E3 Fab'(PEG<sub>5000</sub>)<sub>2</sub>.

Figure 13 is a graph showing dose dependent inhibition of platelet aggregation by c7E3 Fab, c7E3 Fab'(PEG<sub>2,000</sub>)<sub>2</sub> or c7E3 Fab'((PEG<sub>5,000</sub>)<sub>2</sub>)<sub>2</sub>.

Figure 14 is a graph showing dose dependent inhibition of platelet aggregation by c7E3 Fab or c7E3 Fab'((PEG<sub>2,000</sub>)<sub>4</sub>)<sub>2</sub>.

15 Figure 15 is a graph showing dose dependent inhibition of platelet aggregation by c7E3 Fab or c7E3 Fab'((PEG<sub>2,000</sub>)<sub>2</sub>)<sub>2</sub>.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to modified antibodies that specifically bind to one or more human integrin receptors selected from the group consisting of GPIIb/IIIa,  $\alpha_v\beta_3$   
20 and Mac-1. In a preferred embodiment, the modified antibody specifically binds to GPIIb/IIIa expressed on the plasma membrane of platelets, thereby inhibiting the binding of fibrinogen to GPIIb/IIIa and platelet aggregation. Preferably, the antibody which binds GPIIb/IIIa further specifically binds the human integrin receptor  $\alpha_v\beta_3$ .

The antibodies can be polyclonal or monoclonal, and the term "antibody" is  
25 intended to encompass both polyclonal and monoclonal antibodies. The terms polyclonal and monoclonal refer to the degree of homogeneity of an antibody preparation, and are not intended to be limited to particular methods of production. The term "antibody" as used herein also encompasses functional fragments of antibodies,



including fragments of chimeric, humanized, primatized, veneered or single chain antibodies. Functional fragments include antigen binding fragments which bind to an integrin selected from the group consisting of GPIIb/IIIa,  $\alpha_v\beta_3$ , and Mac-1. For example, antibody fragments capable of binding to GPIIb/IIIa and/or  $\alpha_v\beta_3$  or portions thereof, including, but not limited to Fv, Fab, Fab' and F(ab')<sub>2</sub> fragments are encompassed by the invention. Such fragments can be produced by enzymatic cleavage or by recombinant techniques, for example, papain or pepsin cleavage can generate Fab or F(ab')<sub>2</sub> fragments, respectively. Other proteases with the requisite substrate specificity can also be used to generate Fab or F(ab')<sub>2</sub> fragments. In a preferred embodiment, Fab fragments are prepared by achromopeptidase cleavage. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons has been introduced upstream of the natural stop site. For example, a chimeric gene encoding a F(ab')<sub>2</sub> heavy chain portion can be designed to include DNA sequences encoding the CH<sub>1</sub> domain and hinge region of the heavy chain.

Single chain antibodies, and chimeric, humanized or primatized (CDR-grafted), or veneered antibodies, as well as chimeric, CDR-grafted or veneered single chain antibodies, comprising portions derived from different species, and the like are also encompassed by the present invention and the term "antibody". The various portions of these antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques. For example, nucleic acids encoding a chimeric or humanized chain can be expressed to produce a contiguous protein. See, e.g., Cabilly *et al.*, U.S. Patent No. 4,816,567; Cabilly *et al.*, European Patent No. 0,125,023 B1; Boss *et al.*, U.S. Patent No. 4,816,397; Boss *et al.*, European Patent No. 0,120,694 B1; Neuberger, M.S. *et al.*, WO 86/01533; Neuberger, M.S. *et al.*, European Patent No. 0,194,276 B1; Winter, U.S. Patent No. 5,225,539; Winter, European Patent No. 0,239,400 B1; Queen *et al.*, European Patent No. 0 451 216 B1; and Padlan, E.A. *et al.*, EP 0 519 596 A1. See also, Newman, R. *et al.*, *BioTechnology*, 10: 1455-1460 (1992), regarding primatized antibody, and Ladner *et al.*, U.S. Patent No. 4,946,778 and Bird, R.E. *et al.*, *Science*, 242: 423-426 (1988) regarding single chain antibodies.

As used herein, the term "humanized antibody" refers to an antibody or antigen binding fragment thereof comprising portions of immunoglobulins of different origin, wherein at least one portion is of human origin. For instance, a humanized antibody can comprise an antigen-binding region of nonhuman origin (e.g., rodent) and a constant region of human origin (e.g., a human framework region, a human constant region (e.g., C<sub>L</sub>, C<sub>H</sub>1, hinge, C<sub>H</sub>2, C<sub>H</sub>3, C<sub>H</sub>4)). The antigen binding region can be derived from an immunoglobulin of nonhuman origin having the requisite specificity.

In one embodiment, the humanized antibody comprises one or more immunoglobulin chains, comprising a CDR of nonhuman origin (e.g., one or more CDRs derived from an antibody of nonhuman origin) and a framework region derived from a light or heavy chain of human origin (e.g., CDR-grafted antibodies with or without framework changes, including single chain antibodies). In another embodiment, the humanized antibody comprises (a) at least one humanized light chain comprising an antigen binding region derived from the light chain of a nonhuman immunoglobulin of the requisite specificity, and a human light chain constant region, and (b) at least one humanized heavy chain comprising an antigen binding region derived from the heavy chain of said nonhuman immunoglobulin, and a human heavy chain constant region. In one aspect of this embodiment, the humanized antibody is a chimeric antibody, comprising a light chain variable region and a heavy chain variable region of an antibody of nonhuman origin, and a human constant region.

In a preferred embodiment, the humanized antibody can compete with murine 7E3 monoclonal antibody or chimeric 7E3, or an antigen binding fragment thereof, for binding to human GPIIb/IIIa or human  $\alpha_v\beta_3$ . For example, the antigen-binding region of the humanized antibody can be derived from 7E3 monoclonal antibody. In one aspect of this embodiment, the humanized immunoglobulin comprises the light and heavy chain variable regions of the murine 7E3 antibody, and a human constant region. In another aspect, the humanized immunoglobulin comprises CDR1, CDR2 and CDR3 of the light chain and CDR1, CDR2 and CDR3 of the heavy chain of the murine 7E3 antibody, and a human constant region.

As used herein, the term "chimeric antibody" also refers to an antibody or antigen binding fragment thereof comprising portions of immunoglobulins of different origin. None of the portions of immunoglobulins which comprise a chimeric antibody need to be of human origin. For example, a chimeric antibody can comprise an antigen-binding region of nonhuman origin (e.g., rodent) and a constant region of non-human primate origin (e.g., a chimpanzee framework region, a chimpanzee constant region (e.g., C<sub>L</sub>, C<sub>H</sub>1, hinge, C<sub>H</sub>2, C<sub>H</sub>3, C<sub>H</sub>4)).

A humanized or chimeric antibody can comprise a light chain constant region (e.g., C<sub>K</sub>, C<sub>λ</sub>), and a heavy chain constant region (e.g., C<sub>H</sub>1, hinge, C<sub>H</sub>2, C<sub>H</sub>3, C<sub>H</sub>4) of a desired isotype, for instance, IgG (e.g., IgG1, IgG2, IgG3, IgG4), IgA (e.g., IgA1, IgA2), IgM, IgE or IgD. In a particular embodiment, a humanized or chimeric antibody can comprise a heavy chain constant region of mixed isotype (e.g., C<sub>H</sub>1 and hinge of IgG origin and C<sub>H</sub>2 and C<sub>H</sub>3 of IgA origin). Preferably, a humanized or chimeric antibody comprises an IgG heavy chain constant region. Most preferably, a humanized or chimeric antibody comprises an IgG1 heavy chain constant region.

Humanized antibodies can be produced using synthetic or recombinant DNA technology using standard methods or other suitable techniques. Nucleic acid (e.g., cDNA) sequences coding for humanized variable regions can also be constructed using PCR mutagenesis methods to alter DNA sequences encoding a human or humanized chain, such as a DNA template from a previously humanized variable region (see e.g., Kamman, M., *et al.*, *Nucl. Acids Res.*, 17: 5404 (1989)); Sato, K., *et al.*, *Cancer Research*, 53: 851-856 (1993); Daugherty, B.L. *et al.*, *Nucleic Acids Res.*, 19(9): 2471-2476 (1991); and Lewis, A.P. and J.S. Crowe, *Gene*, 101: 297-302 (1991)). Using these or other suitable methods, variants can also be readily produced. In one embodiment, cloned variable regions can be mutagenized, and sequences encoding variants with the desired specificity can be selected (e.g., from a phage library; see e.g., Krebber *et al.*, U.S. 5,514,548; Hoogenboom *et al.*, WO 93/06213, published April 1, 1993)).

Antibodies which are specific for human GPIIb/IIIa,  $\alpha_v\beta_3$  or Mac-1 can be raised against an appropriate immunogen, such as isolated and/or recombinant GPIIb/IIIa,  $\alpha_v\beta_3$  or Mac-1 or portions thereof (including synthetic molecules, such as

synthetic peptides). Antibodies can also be raised by immunizing a suitable host (e.g., mouse) with cells that express GPIIb/IIIa, such as platelets (see e.g., Collier, U.S. Pat. No. 5,440,020, the entire teachings of which are incorporated herein by reference) or cells that express  $\alpha_v\beta_3$  or Mac-1. In addition, cells expressing recombinant GPIIb/IIIa,  $\alpha_v\beta_3$  or Mac-1 such as transfected cells, can be used as immunogens or in a screen for antibody which binds receptor (See e.g., Chuntharapai *et al.*, *J. Immunol.*, 152: 1783-1789 (1994); Chuntharapai *et al.*, U.S. Patent No. 5,440,021).

Preparation of immunizing antigen, and polyclonal and monoclonal antibody production can be performed using any suitable technique. A variety of methods have been described (see e.g., Kohler *et al.*, *Nature*, 256: 495-497 (1975) and *Eur. J. Immunol.* 6: 511-519 (1976); Milstein *et al.*, *Nature* 266: 550-552 (1977); Koprowski *et al.*, U.S. Patent No. 4,172,124; Harlow, E. and D. Lane, 1988, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY); *Current Protocols In Molecular Biology*, Vol. 2 (Supplement 27, Summer '94), Ausubel, F.M. *et al.*, Eds., (John Wiley & Sons: New York, NY), Chapter 11, (1991)). Generally, a hybridoma is produced by fusing a suitable immortal cell line (e.g., a myeloma cell line such as SP2/0 or P3X63Ag8.653) with antibody producing cells. The antibody producing cells, preferably those of the spleen or lymph nodes, can be obtained from animals immunized with the antigen of interest. The fused cells (hybridomas) can be isolated using selective culture conditions, and cloned by limiting dilution. Cells which produce antibodies with the desired specificity can be selected by a suitable assay (e.g., ELISA).

Other suitable methods of producing or isolating antibodies of the requisite specificity can be used, including, for example, methods which select recombinant antibody from a library (e.g., a phage display library), or which rely upon immunization of transgenic animals (e.g., mice) capable of producing a repertoire of human antibodies (see e.g., Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA*, 90: 2551-2555 (1993); Jakobovits *et al.*, *Nature*, 362: 255-258 (1993); Lonberg *et al.*, U.S. Patent No. 5,545,806; Surani *et al.*, U.S. Patent No. 5,545,807; Lonberg *et al.*, WO97/13852).

As used herein the term "specific antibody" or "specific" when referring to an antibody-antigen interaction is used to indicate that the antibody binds a particular antigen with high affinity (e.g.,  $K_D$  less than about  $10^{-5}$  M), rather than to indicate that the antibody binds to only one antigen.

- 5 An antibody that binds to GPIIb/IIIa,  $\alpha_v\beta_3$  and/or Mac-1, and preferably inhibits the binding of fibronectin to GPIIb/IIIa and inhibits platelet aggregation can be modified as described herein. In one embodiment, 7E3 (a murine IgG1, K) or a humanized 7E3 is modified as described herein. A preferred humanized 7E3 is c7E3, which comprises the antigen binding regions of the heavy and light chains of murine
- 10 7E3, and human  $\gamma 1$  and K constant regions (Knight, D.M., *et al.*, *Molecular Immunology*, 32(16): 1271-1281 (1995)). Other humanized 7E3 antibodies which can comprise other constant regions of human origin and/or human framework regions can also be modified as described herein. In preferred embodiments, functional fragments (e.g., Fv, Fab, Fab', F(ab')<sub>2</sub>) of 7E3 or a humanized 7E3 are modified. Other antibodies
- 15 having an epitopic specificity similar to that of 7E3 or c7E3 for GPIIb/IIIa,  $\alpha_v\beta_3$  and/or Mac-1, including antibodies which bind to the same or a functionally equivalent epitope on GPIIb/IIIa,  $\alpha_v\beta_3$  and/or Mac-1 as is bound by c7E3 or 7E3 can be modified. Antibodies with an epitopic specificity which is the same or similar to that of c7E3 or 7E3 include antibodies which can block the binding of the c7E3 or 7E3 or a suitable
- 20 antigen binding fragment thereof (e.g., c7E3 Fab, 7E3 Fab) to GPIIb/IIIa,  $\alpha_v\beta_3$  and/or Mac-1. In one embodiment, the antibody 7E3 (murine hybridoma 7E3 was deposited on May 30, 1985 at the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209, USA, and is available under accession number HB 8832) competitively inhibits the binding of the modified antibody of the invention to
- 25 GPIIb/IIIa and/or  $\alpha_v\beta_3$ .

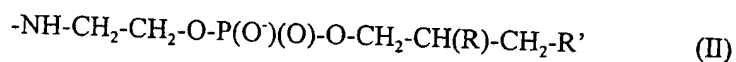
The modification of antibodies as described herein improves the pharmacokinetic properties of the antibody. Specifically, the modified antibodies of the invention are characterized by an increased *in vivo* serum half-life. In a preferred embodiment, the modified antibodies are further characterized by decreased

immunogenicity compared to the unmodified antibody. Assessments of the serum half-life and immunogenicity of proteins and peptides (e.g., antibodies, modified antibodies) can be performed using any suitable method. For example, a modified protein can be administered to an animal (e.g., by injection), and serum can be obtained from the animal at a specified time after administration (e.g., a minute, hour, day, week, month, etc.). The serum can be assayed for the presence of the modified protein or for antibodies which bind to the modified protein (e.g., by ELISA).

In a preferred embodiment, the modification does not significantly affect the antigen-binding properties of the antibody. The antigen-binding properties of an antibody can be expressed as the antigen-antibody association rate constant ( $k_a$ ), the antigen-antibody dissociation rate constant ( $k_d$ ) and affinity ( $K_D$ ). The parameters can be measured experimentally using any suitable method. (See, for example, Berzofsky, *et al.*, "Antibody-Antigen Interactions", In *Fundamental Immunology*, Paul, W. E., Ed., Raven Press: New York, NY (1984); Kuby, Janis *Immunology*, W. H. Freeman and Company: New York, NY (1992); and the method described herein). A modification that does not significantly affect the antigen-binding properties of an antibody will not change (e.g., increase or decrease) the affinity of the antibody by more than a factor of about 100. Such modified antibodies bind to an antigen with an affinity that is substantially the same as the affinity of the corresponding unmodified antibody. For example, an antibody can have an affinity of  $2 \times 10^{-9}$  M and the modified antibody can have an affinity between about  $2 \times 10^{-11}$  M and about  $2 \times 10^{-7}$  M. In a preferred embodiment, the modification will not change the affinity of the antibody by more than a factor of about ten. The measured affinity of a particular antibody-antigen interaction can vary if measured under different conditions (e.g., salt concentration, pH). Thus, measurements of antigen-binding parameters (e.g.,  $k_a$ ,  $k_d$ ,  $K_D$ ) are preferably made with standardized solutions of antibody and antigen, and a standardized buffer, such as the buffer described herein.

The modified antibodies of the invention comprise one or more organic moieties which are covalently bonded, directly or indirectly, to the antibody, thereby increasing the *in vivo* serum half-life of the antibody, and preferably, decreasing the

immunogenicity compared to the unmodified antibody. As used herein, the term “organic moiety” refers to a linear or branched moiety selected from a hydrophilic polymeric group, a fatty acid group, a fatty acid ester group, a lipid group (e.g., diacylglycerol group, sphingolipid group (e.g., ceramidyl)) or a phospholipid group (e.g., phosphatidyl ethanolamine group). Preferred lipid and phospholipid groups are represented by Structural Formulas I and II, respectively.



In Structural Formulas I and II, R and R' are each, independently, -OH or a group represented by -X-Y, wherein -X- is -O-, -O-C(O)-, -S-, -O-S(O)<sub>2</sub>-O-, -NH-C(O)- or -O-CH=CH-, and Y is a hydrocarbon group containing from about 6 to about 40 carbon atoms, which is saturated or which contains one or more units of unsaturation (e.g., alkyl, alkenyl, alkynyl), provided that both R and R' are not -OH. Preferably, -X- is -O-C(O)-. Thus, suitable lipid and phospholipid groups include, for example, diacylglycerols, ether phospholipids, thioether phospholipids, lysophospholipids and plasmalogens.

Each organic moiety that is bonded to an antibody of the invention can independently be a hydrophilic polymeric group, a fatty acid group, a fatty acid ester group, a lipid group or a phospholipid group. As used herein, the term “fatty acid” encompasses mono-carboxylic acids and di-carboxylic acids.

A “hydrophilic polymeric group”, as the term is used herein, refers to an organic polymer which is more soluble in water than in octane. For example, polylysine is more soluble in water than in octane. Thus, an antibody modified by the covalent attachment of polylysine is encompassed by the invention. Hydrophilic polymers suitable for modifying antibodies of the invention can be linear or branched and include, for example, polyalkane glycols (e.g., PEG, monomethoxy-polyethylene glycol (mPEG), PEG diamine, PPG and the like), carbohydrates (e.g., dextran, cellulose,

oligosaccharides, polysaccharides and the like), polymers of hydrophilic amino acids (e.g., polylysine, polyarginine, polyaspartate and the like), polyalkane oxides (e.g., polyethylene oxide, polypropylene oxide and the like) and polyvinyl pyrrolidone. The hydrophilic polymer which modifies the antibody of the invention can have a molecular weight of about 800 to about 150,000 Daltons as a separate molecular entity. Preferably, the organic moiety has a molecular weight of about 2,000 Daltons or more as a separate molecular entity. For example, PEG<sub>5000</sub> and PEG<sub>20,000</sub>, wherein the subscript is the average molecular weight of the polymer in Daltons, are particularly preferred hydrophilic polymers. Also preferred are PEG<sub>2000</sub>, PEG<sub>3,400</sub>, PEG<sub>10,000</sub>, PEG<sub>30,000</sub> and PEG<sub>40,000</sub> which can be linear or branched. Branched PEGs (e.g., (PEG<sub>2,000</sub>)<sub>2</sub>, (PEG<sub>2,000</sub>)<sub>4</sub>, (PEG<sub>5,000</sub>)<sub>2</sub>) can be prepared, for example, as described by Harris *et al.*, U.S. Patent No. 5,932,462, the entire teachings of which are incorporated herein by reference.

In one embodiment, the hydrophilic polymeric group is substituted with one to about six alkyl, fatty acid, fatty acid ester, lipid or phospholipid groups (as described herein, e.g., Formulas I and II). Preferably, the substituted hydrophilic polymeric group is a linear or branched PEG. More preferably, the substituted hydrophilic polymeric group is a linear PEG (e.g., PEG diamine) that is terminally substituted with a fatty acid, fatty acid ester, lipid or phospholipid group. Hydrophilic polymers that are substituted with an alkyl, fatty acid, fatty acid ester, lipid or phospholipid groups group can be prepared using suitable methods. For example, as described herein (Examples XIV and XV), a modifying agent can be prepared by reacting monoprotected PEG diamine with an activated fatty acid (e.g., palmitoyl chloride). The resulting product can be deprotected, a suitable activating group can be introduced (e.g., maleimido), and the resulting modifying agent can be used to produce a modified Fab' which comprises a PEG that is terminally substituted with a fatty acid group. A variety of other suitable synthetic schemes can be used, for example, a polymer comprising an amine group can be coupled to a carboxylate of the fatty acid or fatty acid ester as described herein, and an activated carboxylate (e.g., activated with N,N'-carbonyl diimidazole) on a fatty acid or fatty acid ester can be coupled to a hydroxyl group on a polymer.



Fatty acids and fatty acid esters suitable for modifying antibodies of the invention can be saturated or can contain one or more units of unsaturation. In a preferred embodiment, the fatty acids and fatty acid esters comprise from about six to about forty carbon atoms or about eight to about forty carbon atoms. Fatty acids which are suitable for modifying antibodies of the invention include, for example,

5 n-dodecanoate ( $C_{12}$ , laurate), n-tetradecanoate ( $C_{14}$ , myristate), n-hexadecanoate ( $C_{16}$ , palmitate), n-octadecanoate ( $C_{18}$ , stearate), n-eicosanoate ( $C_{20}$ , arachidate), n-docosanoate ( $C_{22}$ , behenate), n-triacontanoate ( $C_{30}$ ), n-tetracontanoate ( $C_{40}$ ), *cis*- $\Delta^9$ -octadecanoate ( $C_{18}$ , oleate), all *cis*- $\Delta^{5,8,11,14}$ -eicosatetraenoate ( $C_{20}$ , arachidonate),

10 octanedioic acid, tetradecanedioic acid, octadecanedioic acid, docosanedioic acid, and the like. Suitable fatty acid esters include mono-esters of dicarboxylic acids which comprise a linear or branched lower alkyl group. The lower alkyl group can comprise from one to about twelve, preferably one to about six, carbon atoms. Suitable fatty acid esters for modifying antibodies of the invention include, for example, methyl

15 octadecanoate, ethyl octadecanoate, propyl octadecanoate, butyl dodecanoate, *sec*-butyl dodecanoate, *tert*-butyl dodecanoate, neopentyl tetradecanoate, hexyl tetradecanoate, methyl *cis*- $\Delta^9$ -octadecanoate, and the like.

The modification of antibodies as described herein below is preferably performed using one or more modifying agents. A "modifying agent" as the term is

20 used herein, refers to a derivative of a hydrophilic polymer, a fatty acid, a fatty acid ester, a lipid or a phospholipid which comprises an activating group. An "activating group" is a chemical moiety or functional group that can, under appropriate conditions, react with a second chemical group thereby forming a covalent bond between the modifying agent and the second chemical group. For example, amine-reactive

25 activating groups include electrophilic groups such as tosylate, mesylate, halo (chloro, bromo, fluoro, iodo), N-hydroxysuccinimidyl esters (NHS), acyl halides and the like. Activating groups which can react with thiols include, for example, maleimide, iodoacetyl, acryloyl, pyridyl disulfides, 5-thiol-2-nitrobenzoic acid thiol (TNB-thiol), and the like. An aldehyde functional group can be coupled to amine- or hydrazide-

containing molecules, and an azide group can react with a trivalent phosphorous group to form phosphoramidate or phosphorimide linkages. Suitable methods to introduce activating groups into molecules are known in the art (see for example, Hermanson, G. T., *Bioconjugate Techniques*, Academic Press: San Diego, CA (1996)). An activating group can be bonded directly to the hydrophilic polymer, fatty acid, fatty acid ester, lipid or a phospholipid or through a linker moiety, for example a C<sub>1</sub>-C<sub>12</sub> hydrocarbyl group. As used herein, "hydrocarbyl group" refers to a hydrocarbon chain wherein one or more carbon atoms is optionally replaced by a heteroatom such as oxygen, nitrogen or sulfur. Suitable linker moieties include, for example, tetraethylene glycol, -(CH<sub>2</sub>)<sub>3</sub>-, -NH-(CH<sub>2</sub>)<sub>6</sub>-NH-, -(CH<sub>2</sub>)<sub>2</sub>-NH- and -CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-NH-.

Modifying agents which comprise a linker moiety can be produced, for example, by reacting a mono-Boc-alkyldiamine (e.g., mono-Boc-ethylenediamine, mono-Boc-diaminohexane) with a fatty acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to form an amide bond between the free amine and the fatty acid carboxylate. The Boc protecting group can be removed from the product by treatment with trifluoroacetic acid (TFA) to expose a primary amine which can be coupled to another carboxylate as described, or can be reacted with maleic anhydride and the resulting product cyclized to produce an activated maleimido derivative of the fatty acid. (See, for example, Thompson, *et al.*, WO 92/16221 the entire teachings of which are incorporated herein by reference.)

The modified antibodies of the invention can be produced by reacting an appropriate antibody (i.e. antibody or suitable antibody fragment, such as Fab') with a modifying agent, as described herein. In one embodiment, the organic moieties can be bonded to the antibody in a non-site specific manner by employing an amine-reactive modifying agent, for example, an NHS ester of PEG. In a specific example, c7E3 Fab was modified by reaction with NHS-PEG<sub>5,000</sub> in a manner which was not site specific. As shown herein, this type of modification substantially decreased the binding affinity of the Fab for GPIIb/IIIa (Example XXII, Table 6, compare c7E3 Fab and c7E3 Fab Random (PEG<sub>5,000</sub>)). Thus, in preferred embodiments, the modified antibodies can be produced by reacting an appropriate antibody with one or more modifying agents to

produce a modified antibody comprising one to about six organic moieties which are bonded to specific sites on the antibody, for example a (i.e., one or more) carboxyl-terminus and/or the side chain sulfur atom of a (i.e., one or more) cysteinyl residue. In particularly preferred embodiments, the modified antibody can comprise one or two  
5 linear PEG moieties of greater than 2,000 Daltons which are bonded to specific sites (e.g., cysteinyl residue which forms an inter-chain disulfide bond, carboxyl terminus of the heavy chain) on the antibody.

In embodiments wherein the modified antibody comprises an organic moiety which is bonded to a cysteinyl residue, it is preferred that the organic moiety is bonded  
10 to a cysteinyl residue in the hinge region of the heavy chain that forms an inter-chain disulfide bond in the properly folded (i.e., native) antibody. As used herein, the term "inter-chain disulfide bond", refers to a disulfide bond which links two heavy chains in a properly folded (i.e., native) antibody.

Modified antibodies of this type can be prepared by reducing the inter-chain  
15 disulfide bonds, thereby producing monovalent antibodies comprising a heavy chain and a light chain. Suitable reducing agents include, for example, 2-mercaptoethanol (2-ME), dithiothreitol (DTT), 2-mercaptoethylamine (2-MEA) and cysteine. Such monovalent antibodies can be isolated and purified by suitable methods (e.g., column chromatography) and the purified antibody can be reacted with a thiol-reactive  
20 modifying agent, for example, O-(2-maleimidoethyl)-O'-methylpolyethylene glycol 5000, to produce the modified antibody of the invention. In one example, the modified antibody comprises a (e.g., one or more) PEG moiety which is bonded to the side chain sulfur atom of at least one of the cysteinyl residues contained within the hinge region of the heavy chain that form an inter-chain disulfide bond.

25 In another example, the modified antibody is a Fab' which comprises an organic moiety which is bonded to the side chain sulfur atom of at least one of the hinge region cysteinyl residues that form an inter-chain disulfide bond.

In another example, the modified antibody is an Fab comprising an organic moiety which is covalently attached to the carboxyl-terminus of the heavy chain.

30 Modified antibodies of this type can be prepared by reacting an Fab generated by papain

cleavage of an IgG with, for example, a PEG comprising an amine activating group in the presence of EDC. However, the amine-activated PEG can react with all carboxyl groups on the antibody making it difficult to purify the antibodies comprising a PEG which is specifically bonded to the carboxyl-terminus of the heavy chain.

5 A preferred method for preparing the modified antibody is to introduce a unique activating group at the carboxyl-terminus of the heavy-chain through reverse proteolysis. "Reverse proteolysis" is a term of the art which refers to the fact that under particular conditions, certain proteases can catalyze the formation of peptide (amide) bonds. For example, a purified Fab generated by the activity of a protease can be mixed  
10 with said protease and carbohydrazide under conditions suitable for reverse proteolysis (e.g., 250-fold molar excess of the carbohydrazide relative to the Fab), to produce an Fab comprising a unique hydrazide function at the carboxyl-terminus of the heavy chain.

The hydrazide containing Fab can be modified by reaction with a suitable  
15 modifying agent. For example, a Fab can be reacted with a modifying agent comprising an aldehyde functional group to produce a modified antibody comprising an organic moiety that is specifically attached to the carboxyl-terminus of the heavy chain through a hydrazone-linkage. The hydrazone can be further stabilized by reaction with a suitable reducing agent (e.g., sodium cyanoborohydride).

20 Various enzymes which can cleave an antibody to produce a desired fragment can be used to introduce a hydrazide function by reverse proteolysis. The conditions for reverse proteolysis are known to those skilled in the art and include a large (e.g., 250-fold) molar excess of carbohydrazide and an extended reaction time. Additionally, the reverse proteolysis reaction may preferentially occur at a pH different from the optimal  
25 pH for proteolysis (Fisch *et al.*, *Bioconjugate Chem.*, 3:147-153 (1992); Werlen *et al.*, *Bioconjugate Chem.*, 5:411-417 (1994); Kumaran *et al.*, *Protein Sci.* 6(10):2233-2241 (1997); Itoh *et al.*, *Bioorg. Chem.*, 24(1): 59-68 (1996); Capellas *et al.*, *Biotechnol. Bioeng.*, 56(4):456-463 (1997)). The optimal pH for reverse proteolysis can be determined empirically using standard methods. A preferred enzyme for carboxyl-  
30 terminus modification is achromopeptidase.

The carboxyl-terminus of a light chain can also be modified by the introduction of a hydrazide group by reverse proteolysis. In one embodiment, a protease that can cleave the light chain without significantly affecting the antigen-binding characteristics of the antibody is selected and the light chain modified as described.

5 In another embodiment, an achromopeptidase or other proteolytic cleavage site can be introduced into the light chain of a cloned antibody employing recombinant DNA technology. This type of antibody can be cleaved by achromopeptidase or a suitable enzyme to generate a Fab wherein a hydrazide group can be introduced at the carboxyl-terminus of both the heavy chain and the light chain by reverse proteolysis.

10 In additional embodiments, recombinant DNA technology can be used to introduce or remove achromopeptidase or other cleavage sites from the heavy chain of a cloned antibody (e.g., by mutagenesis). For example, the naturally occurring achromopeptidase cleavage site can be removed and a new cleavage site introduced at a different position. Thus, achromopeptidase or other suitable enzymes can be used to  
15 generate a variety of modified antibodies of the invention. For example, Fv, Fab, F(ab')<sub>2</sub> or Fab' fragments comprising an organic moiety which is bonded to the carboxyl-terminus of the heavy chain and/or the carboxyl-terminus of the light chain can be produced. Such fragments can further comprise one or more additional organic moieties which are bonded to the side chain sulfur atoms of cysteinyl residues.

20 In a particular embodiment, the modified antibody is a c7E3 Fab' comprising two PEG<sub>5,000</sub> groups, wherein each PEG<sub>5,000</sub> group is bonded to the side chain sulfur atom of a cysteinyl residue that forms an inter-chain disulfide bond.

In another particular embodiment, the modified antibody is a c7E3 Fab' comprising two PEG<sub>20,000</sub> groups, wherein each PEG<sub>20,000</sub> group is bonded to the side  
25 chain sulfur atom of a cysteinyl residue that forms an inter-chain disulfide bond.

In another particular embodiment, the modified antibody is a c7E3 Fab comprising a PEG<sub>5,000</sub> group which is bonded to the carboxyl-terminus of the heavy chain. In a preferred aspect, the Fab is generated by achromopeptidase cleavage.

In another particular embodiment, the modified antibody is a c7E3 Fab comprising a PEG<sub>20,000</sub> group which is bonded to the carboxyl-terminus of the heavy chain. In a preferred aspect, the Fab is generated by achromopeptidase cleavage.

In another particular embodiment, the modified antibody is a c7E3 Fab' comprising a PEG<sub>5,000</sub> group and a C<sub>22</sub> fatty acid group which are each independently bonded to the side chain sulfur atom of a cysteinyl residue that forms an inter-chain disulfide bond.

In another particular embodiment, the modified antibody is a c7E3 Fab comprising a PEG<sub>5,000</sub> group which is bonded to the carboxyl-terminus of the heavy chain, wherein the PEG<sub>5,000</sub> bears a C<sub>22</sub> fatty acid substituent.

In another particular embodiment, the modified antibody is a c7E3 Fab' comprising a (i.e., one or more) PEG<sub>2,000</sub> group which is bonded to the side chain sulfur atom of a cysteinyl residue that forms an inter-chain disulfide bond in the hinge region.

In another particular embodiment, the modified antibody is a c7E3 Fab' comprising a (i.e., one or more) PEG<sub>10,000</sub> group which is bonded to the side chain sulfur atom of a cysteinyl residue that forms an inter-chain disulfide bond in the hinge region.

In another particular embodiment, the modified antibody is a c7E3 Fab' comprising a (i.e., one or more) (PEG<sub>2,000</sub>)<sub>2</sub> group which is bonded to the side chain sulfur atom of a cysteinyl residue that forms an inter-chain disulfide bond in the hinge region.

In another particular embodiment, the modified antibody is a c7E3 Fab' comprising a (i.e., one or more) (PEG<sub>2,000</sub>)<sub>4</sub> group which is bonded to the side chain sulfur atom of a cysteinyl residue that forms an inter-chain disulfide bond in the hinge region.

In another particular embodiment, the modified antibody is a c7E3 Fab' comprising a (i.e., one or more) (PEG<sub>5,000</sub>)<sub>2</sub> group which is bonded to the side chain sulfur atom of a cysteinyl residue that forms an inter-chain disulfide bond in the hinge region.

In another particular embodiment, the modified antibody is a c7E3 Fab' comprising a (i.e., one or more) terminally substituted PEG<sub>3,400</sub> group which is bonded

to the side chain sulfur atom of a cysteinyl residue that forms an inter-chain disulfide bond in the hinge region, wherein the terminal substituent is a palmitoyl group.

In still another particular embodiment, the modified antibody is a c7E3 Fab comprising a C<sub>22</sub> fatty acid group, which is bonded to the carboxyl-terminus of the  
5 heavy chain.

In an additional embodiment, an antibody comprising an engineered cysteinyl residue (e.g., a cysteinyl residue that was introduced through recombinant DNA technology) is modified. Antibodies of this type, including, for example, Fv, Fab, Fab', F(ab')<sub>2</sub> fragments, can be readily prepared by those of ordinary skill in the art. The  
10 antibody comprising an engineered cysteinyl residue can be reacted with a thiol-reactive modifying agent to produce the modified antibody of the invention. The antibody comprising an engineered cysteinyl residue can further comprise an achromopeptidase or other cleavage site and be modified at this site as described.

The site specific modification (e.g., carboxyl-terminal PEGylation) of antibodies  
15 as described herein provides several advantages including an increased duration of action. Further, antibodies comprising site specific modifications can unexpectedly be less immunogenic than the corresponding unmodified antibodies. For example, site specific modification of Fab fragments with one or two low molecular weight organic moieties (e.g., PEG<sub>5000</sub>) produces a modified Fab with an increased serum half-life and  
20 significantly decreased immunogenicity, that binds to GPIIb/IIIa or  $\alpha_v\beta_3$  with an affinity that is substantially the same as the unmodified antibody (see examples IX, XI and XII). The unexpected benefit of a remarkable decrease in immunogenicity as a result of limited modification is likely due to the site-specific nature of the modification described herein.

25 The modified antibodies of the invention can be purified by employing methodologies which are known to those of skill in the art. Suitable purification methods can include, for example, column chromatography (e.g., ion-exchange, gel filtration, hydrophobic-interaction, affinity), preparative native electrophoresis, precipitation and ultrafiltration. Preferably, the modified antibodies are purified by  
30 hydrophobic-interaction chromatography as described herein.

As used herein, the term "pure" or "purified" refers to a preparation wherein the modified antibody of the invention accounts for about 75% of the macromolecules present in the preparation. Preferably the modified antibody accounts for about 90% or 95% of the macromolecules present in the preparation. It is most preferred that the  
5 modified antibody accounts for about 99% or essentially all macromolecules present in the preparation.

In additional embodiments, the invention provides a method of treating (e.g., reducing the severity of or preventing) a disease or disorder in which cells expressing GPIIb/IIIa,  $\alpha_v\beta_3$  and/or Mac-1 (e.g., platelets, endothelial cells, leukocytes) play a  
10 pathophysiological role in a human. Such disorders include, for example, thromboembolic disorders such as cardiovascular disease (e.g., atherosclerosis, coronary artery disease, angina, myocardial infarction), deep vein thrombosis, stroke, ischemia, tumor vascularization (e.g.,  $\alpha_v\beta_3$ -mediated angiogenesis), tumor metastasis (e.g., platelet-mediated metastasis). Cells which express GPIIb/IIIa,  $\alpha_v\beta_3$  and/or Mac-1 (e.g.,  
15 platelets, endothelial cells, leukocytes) also play a pathophysiological role in inflammatory diseases and conditions, which include, for example, autoimmune diseases, adult respiratory distress syndrome, allograft rejection, sepsis, reperfusion injury (e.g., transplantation associated reperfusion injury, injury from reperfusion in stroke or myocardial infarction patients), myocardial infarction, angioplasty and  
20 surgery.

The method of the invention can also be used to beneficially inhibit thrombosis, stenosis, restenosis and/or a process mediated by the binding of a ligand to GPIIb/IIIa,  $\alpha_v\beta_3$  and/or Mac-1 (e.g., adhesion, aggregation, secretion (e.g., degranulation), proliferation, generation of oxygen radicals) in a human. For example, the modified  
25 antibodies find use in a situation where thrombus formation or reformation or stenosis and/or restenosis is to be prevented, such as, during or following a vascular intervention procedure, for example, angiography, angioplasty (e.g., performed by balloon, atherectomy, laser angioplasty or other suitable methods (with or without rotablation and/or stent placement)), coronary artery by-pass surgery, stent placement (e.g.,  
30 coronary stent), and/or other vascular intervention procedures (e.g., vascular surgery,



vascular graft, deployment of a peripheral stent, insertion of a prosthetic valve or vessel (e.g., in autologous, non-autologous or synthetic vessel graft)). In particular, the method can be used to beneficially inhibit thrombosis during or following a coronary artery intervention procedure, such as percutaneous transluminal coronary angioplasty (PTCA).

The method comprises the administration of an effective amount of a modified antibody of the invention to a human to prevent or decrease the severity of a disease and/or disorder in which platelets play a pathophysiological role, or to inhibit (reduce or prevent) thrombosis, stenosis, restenosis and/or a process mediated by the binding of a ligand to GPIIb/IIIa,  $\alpha_v\beta_3$  and/or Mac-1. For example, the modified antibody can be administered to a human before, during and/or after a vascular intervention procedure (e.g., angioplasty) to prevent abrupt closure of the vessel (reocclusion) and prevent/or reduce acute ischemia.

An "effective amount", as the term is used herein, is a quantity that is sufficient to achieve a desired therapeutic effect (e.g., inhibit thrombus formation, prevent vessel reocclusion, inhibit stenosis and/or restenosis, inhibit inflammation, inhibit  $\alpha_v\beta_3$ -mediated angiogenesis).

In one embodiment, the method of the invention is a method of inhibiting thrombosis in a human comprising administering to said human an effective amount of a modified antibody of the invention.

In another embodiment, the method of the invention is a method of inhibiting stenosis and/or restenosis following a vascular intervention procedure (e.g., coronary artery intervention procedure such as PTCA) in a human comprising administering to said human an effective amount of a modified antibody of the invention.

In another embodiment, the method of the invention is a method of reducing or preventing ischemia in a human comprising administering to said human an effective amount of a modified antibody of the invention.

In another embodiment the method of the invention is a method of inhibiting the growth and/or metastasis of a tumor in a human comprising administering to said human an effective amount of a modified antibody of the invention.

In another embodiment, the invention provides a method of inhibiting  $\alpha_v\beta_3$ -dependent angiogenesis in a human comprising administering an effective amount of a modified antibody of the invention to the human.

5 In another embodiment, the method of the invention is a method of inhibiting a process mediated by the binding of a ligand to GPIIb/IIIa,  $\alpha_v\beta_3$  and/or MAC-I in a human comprising administering to said human a therapeutically effective amount of modified antibody of the invention.

The method of the invention includes the administration of one or more modified antibodies alone or in combination with an effective amount of one or more additional active agents, for example, a fibrinolytic agent (e.g., Retavase), a thrombolytic agent, such as a plasminogen activator (e.g., tissue plasminogen activator, urokinase, streptokinase, recombinant plasminogen activator), anticoagulant (e.g., heparin, hirulog, hirudin, aspirin), or a coumarin anticoagulant (e.g., warfarin, ethyldine dicoumarol). The modified antibodies of the invention can also be administered with a  $\beta$ -adrenergic blocker (e.g., alprenolol, acebutolol, propanolol), calcium channel blocker (e.g., nifedipine, diltiazem, cinnarizine, bencyclane) or vasodilator (e.g., nitroglycerin, amotriphene, erythritol, prenylamine). The modified antibodies of the invention can also be administered in combination with an agent which stimulates the production of nitric oxide (see, for example, Singh *et al.*, U.S. Pat. No. 20 5,811,437).

A variety of routes of administration are possible. For example, the modified antibodies of the invention can be prepared and administered in the same manner as has been described for other therapeutic antibodies (Faanes *et al.*, U.S. Pat. No. 5,695,760; Coller *et al.*, WO 95/12412; both hereby incorporated by reference). An effective amount of a modified antibody of the invention can be administered parenterally (e.g., intravenously, intraarterially, intramuscularly, subcutaneously), for example, by injection into a blood vessel, muscle or cavity (e.g., peritoneal cavity, thoracic cavity). The modified antibodies can also be administered orally, topically, by inhalation (e.g., intrabronchial, intranasal, oral inhalation or intranasal drops), or rectally. Preferably,

the modified antibodies are administered intravenously. The modified antibody can be administered in a single dose, continuous infusion, or in multiple doses and/or infusions (e.g., a bolus dose followed by continuous infusion).

Formulation of an antibody to be administered will vary according to the route of administration selected (e.g., solution, emulsion, capsule). The modified antibodies of the invention can be administered as neutral proteins or as physiologically acceptable salts. By the term "salt" it is meant that the charged groups on the protein (e.g., protonated amines, carboxylates) are associated with an exchangeable ion of opposite charge. For example, positively charged amino groups (e.g., protonated amines) can form acid addition salts with inorganic acids (e.g., hydrochloric acid, phosphoric acid, methane sulfonic acid, and the like) or organic acids (e.g., acetic acid, lactic acid, citric acid and the like), and contain a counterion such as  $\text{Cl}^-$ ,  $\text{CH}_3\text{SO}_3^-$ , acetate, lactate, citrate, and the like. Salts of carboxylates can be formed by reaction with a suitable base such as a hydroxide base (e.g., sodium hydroxide, potassium hydroxide), and contain a counterion such as  $\text{Na}^+$ ,  $\text{K}^+$ , and the like.

The modified antibody can be administered as part of a pharmaceutical composition comprising a modified antibody and a pharmaceutically acceptable vehicle or carrier. Suitable pharmaceutical carriers may contain inert ingredients which do not interact with the modified antibody. Standard pharmaceutical formulation techniques can be employed, such as those described in *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, PA. Suitable pharmaceutical carriers for parenteral administration include, for example, sterile water, physiological saline, bacteriostatic saline (saline containing about 0.9% mg/ml benzyl alcohol), phosphate-buffered saline, Hank's solution, Ringer's-lactate and the like. The antibody formulation may contain additional additives, such as a stabilizer (e.g., Polysorbate 80, USP/NP). Methods for encapsulating compositions (such as in a coating of hard gelatin or cyclodextran) are known in the art (Baker, *et al.*, *Controlled Release of Biological Active Agents*, John Wiley and Sons, (1986)).

The amount of antibody to be administered to the human can depend on the type and severity of the disease and on the characteristics of the human, such as general health, age, sex and body weight and tolerance to drugs. It can also depend

on the degree, severity and type of disease. The skilled artisan will be able to determine appropriate dosages depending on these and other factors. Typically, a therapeutically effective amount of the modified antibody can range from about 0.01 mg/kg to about 100 mg/kg for a bolus and about 0.001 mg/min to about 1 mg/min for a continuous infusion, although larger or smaller quantity can be used.

#### EXEMPLIFICATION

##### Example I Synthesis of c7E3 Fab' (PEG<sub>5,000</sub>)<sub>2</sub>

18 ml of c7E3 F(ab')<sub>2</sub> ( 6 mg/ml in phosphate buffered saline, prepared by pepsin digestion of c7E3 IgG) was treated with 1.8 ml of dithiothreitol (16 mg/ml) and allowed to stand at room temperature for one hour. 1.6 g of ammonium sulfate were dissolved in the solution , and the Fab' solution was applied to a TosoHaas phenyl-5PW column (21.5 mm X 15 cm, 13μm) which had been equilibrated with 0.1 M sodium phosphate pH 7.5 containing 0.6 M ammonium sulfate. The Fab' was eluted by reducing the ammonium sulfate concentration to 0 over 65 minutes with a flow rate of 6 ml/min. Fractions containing the Fab' were pooled and concentrated in an Amicon stirred cell to a final concentration of 2 mg/ml. A total of 74 mg of the Fab' was obtained. The Fab' was then treated with 2 ml of an aqueous solution of O-(2-maleimidoethyl)-O'-methylpolyethylene glycol 5000 (66.5 mg/ml; 20 eq total) (Shearwater Polymers, Huntsville, Al). After two hours 3.2 g of ammonium sulfate were added and dissolved in the Fab' solution, and the solution was applied to the phenyl column equilibrated with 0.1 M sodium phosphate pH 7.5 containing 0.6 M ammonium sulfate. The PEGylated product was eluted in a 30 to 80% gradient of 0.1 M sodium phosphate pH 7.5 over 65 minutes with a flow rate of 6 ml/min. Pure fractions were combined to yield 45 mg of product. The average molecular weight was determined to be 59665 Daltons by mass spectrometry (MALDI-TOF).

##### Example II Synthesis of c7E3 Fab' (PEG<sub>20,000</sub>)<sub>2</sub>

7 ml of c7E3 F(ab')<sub>2</sub> ( 5.2 mg/ml in phosphate buffered saline) were treated with 0.7 ml of dithiothreitol (16 mg/ml) and allowed to stand at room temperature for one hour. 0.63 g Ammonium sulfate were dissolved in the solution and the Fab'

solution was applied to a Tosohaas phenyl-5PW column (21.5 mm X 15 cm, 13  $\mu$ m) which had been equilibrated with 0.1 M sodium phosphate pH 7.5 containing 0.6 M ammonium sulfate. The Fab' was eluted by reducing the ammonium sulfate concentration to 0 over 65 minutes with a flow rate of 6 ml/min. Fractions  
5 containing the Fab' were pooled and concentrated in an Amicon stirred cell to a final concentration of 2.2 mg/ml. A total of 22 mg of the Fab' was obtained. The Fab' was then treated with 2 ml of an aqueous solution of O-(2-maleimidoethyl)-O'-methylpolyethylene glycol 20000 (57 mg/ml, 13 eq total)(Shearwater Polymers, Huntsville, Al). After two hours, 0.95 g of ammonium sulfate were dissolved in the  
10 solution, and the solution was applied to the phenyl column equilibrated with 0.1 M sodium phosphate pH 7.5 containing 0.6 M ammonium sulfate. The PEGylated product was eluted using a 50 to 100% gradient of 0.1 M sodium phosphate pH 7.5 over 65 minutes with a flow rate of 6 ml/min. Pure fractions were combined to yield 13 mg of product. The average molecular weight was determined to be 91078  
15 Daltons by mass spectrometry (MALDI-TOF).

#### Example III Synthesis of c7E3 Fab-PEG<sub>5,000</sub>

To 5 ml of c7E3 Fab (7.3 mg/ml) prepared by the action of achromopeptidase on c7E3 IgG was added 1.0 g of carbohydrazide. The pH was adjusted to 4.95 by the addition of 300  $\mu$ l of glacial acetic acid. 10,000 Units of  
20 achromopeptidase were added and the resulting solution incubated at ambient temperature for 24 hours. The solution was dialyzed into 10 mM sodium acetate, pH 4.5. 0.875 mg of monomethoxypolyethylene glycol aldehyde (PEG<sub>5,000</sub> aldehyde, MW = 5,000) (Shearwater Polymers, Huntsville, Al) were added, followed by the addition of 0.3 mg of sodium cyanoborohydride. The reaction was  
25 allowed to stand at ambient temperature overnight. Ammonium sulfate was added to make the final solution 0.6 M in ammonium sulfate. A Tosohaas TSK 5PW phenyl column (21.5 mm x 15 cm) was equilibrated with 100 mM sodium phosphate, pH 7.5 containing 0.6 M ammonium sulfate (buffer A). The reaction mixture was injected onto the phenyl column and eluted with a 60 minute linear  
30 gradient of buffer A to 100 mM sodium phosphate, pH 7.5 (buffer B) at a flow rate of 6 ml/min. The peak eluting at approximately 58 minutes was collected,

concentrated to 5 ml by ultrafiltration at 40 psi with a membrane having a molecular weight exclusion of 10,000 Daltons. The concentrate was dialyzed into phosphate buffered saline (PBS). A small sample was desalted on a PD-10 column, eluting with water. Mass spectral analysis gave a cluster of peaks (due to the molecular weight dispersion of the PEG<sub>5,000</sub> aldehyde) centered at 52,846 Daltons, corresponding to the expected molecular weight of the c7E3 Fab after the addition of carbonylhydrazide and PEG<sub>5,000</sub> aldehyde.

Example IV Inhibition of Platelet Aggregation by c7E3 Fab'(PEG<sub>5,000</sub>)<sub>2</sub>  
and c7E3 Fab'(PEG<sub>20,000</sub>)<sub>2</sub>

10 Preparation of Platelet Rich Plasma (PRP) and Platelet Count Adjustment

All studies were performed utilizing platelets obtained from normal, non-medicated human donors. Blood from each donor was drawn via venipuncture into a 60 ml syringe containing 1/100 final dilution of 40% trisodium citrate. PRP was prepared by centrifugation at 600 g for 4 minutes at room temperature. The PRP was carefully removed and platelet poor plasma (PPP) was prepared by centrifugation of the remaining blood at 2500 g for 20 minutes at room temperature. PRP was allowed to rest at room temperature for 20 minutes prior to counting. PRP platelet counts were determined using a Coulter T-890. The platelet counts were adjusted to 200,000- 250,000 platelets per  $\mu$ l using autologous PPP.

20 Platelet Aggregation

Platelet aggregation assays were performed using a PAP-4D platelet aggregometer. For each assay, 450  $\mu$ l of PRP was added to an aggregometer cuvette containing a stir bar. Serial dilutions of c7E3 Fab (ReoPro®, Centocor, Inc., Malvern, PA), c7E3, c7E3 Fab'(PEG<sub>5,000</sub>)<sub>2</sub> and c7E3 Fab'(PEG<sub>20,000</sub>)<sub>2</sub> starting at 20  $\mu$ g/ml (final concentration) or saline (50  $\mu$ l) were added to each cuvette and incubated at 37°C for 10 minutes prior to the start of the run. The cuvettes were then transferred to the aggregometer channels and the stir speed was adjusted to 1200 rpm. Baseline aggregations were monitored for one-half to one minute prior to the addition of 15-20  $\mu$ M adenosine diphosphate (ADP). Aggregations were monitored

for 4 minutes after the addition of agonist, a control sample, treated with saline only, was included in each run to determine maximal agonist induced platelet aggregation.

#### Inhibition of Aggregation

Inhibition of aggregation is expressed as a percentage of maximum aggregation of agonist stimulated platelets in the absence of c7E3 Fab, c7E3 Fab'(PEG<sub>5,000</sub>)<sub>2</sub> or c7E3 Fab'(PEG<sub>20,000</sub>)<sub>2</sub>. The extent of inhibition of aggregation for each antibody concentration was calculated as follows:

$$\% \text{ Inhibition} = \left[ 1 - \frac{\% \text{ light transmission antibody}}{\% \text{ light transmission saline control}} \right] \times 100$$

The c7E3 Fab, c7E3 Fab'(PEG<sub>5,000</sub>)<sub>2</sub> and c7E3 Fab'(PEG<sub>20,000</sub>)<sub>2</sub> final concentrations were mathematically converted to molar concentrations and plotted versus % inhibition of aggregation. c7E3 Fab, c7E3 Fab'(PEG<sub>5,000</sub>)<sub>2</sub> and c7E3 Fab'(PEG<sub>20,000</sub>)<sub>2</sub> samples demonstrated a similar ability to inhibit ADP mediated platelet aggregation to within a two-fold molar IC<sub>50</sub> concentration.

Figures 1 and 2 show that the c7E3 Fab'(PEG<sub>5,000</sub>)<sub>2</sub> and c7E3 Fab'(PEG<sub>20,000</sub>)<sub>2</sub> are similar to c7E3 Fab in their ability to inhibit platelet aggregation and that the chemical modifications to increase half-life do not affect activity.

Example V Competition binding of c7E3 Fab'-(PEG<sub>20,000</sub>)<sub>2</sub> with <sup>125</sup>I-c7E3 Fab to purified integrins

Purified α<sub>v</sub>β<sub>3</sub> (Smith *et al.*, *J. Biol. Chem.* 263(35):18726-18731 (1988)) or GPIIb/IIIa (Pytela *et al.*, *Science* 231(4745):1559-1562 (1986)) was diluted in Tris-buffered saline (TBS) containing 2 mM calcium chloride (TBS/Ca) to a concentration of 0.5 μg/ml (α<sub>v</sub>β<sub>3</sub>) or 1.5 μg/ml (GPIIb/IIIa). 96-well polystyrene Immulon Removawell plates (Dynex Technologies, Chantilly, VA) were coated with α<sub>v</sub>β<sub>3</sub> or GPIIb/IIIa by aliquoting 50μl of the appropriate protein solution into each well and incubating the plate at room temperature for 2.5 hours. Plates were washed and blocked with 1% bovine serum albumin (BSA) in TBS/Ca for 1 hour at room temperature. c7E3 Fab (ReoPro<sup>®</sup>, Centocor, Inc., Malvern, PA) was iodinated using

Iodobeads® (Pierce Chemicals, Rockford, IL) to approximately 2  $\mu\text{Ci}/\mu\text{g}$ . Dilution series of unlabeled c7E3 Fab'-(PEG<sub>20,000</sub>)<sub>2</sub> and unlabeled c7E3 Fab were prepared in TBS/BSA/Ca at 2X final concentration. Dilutions of c7E3 Fab'-(PEG<sub>20,000</sub>)<sub>2</sub> or c7E3 Fab (starting at 20  $\mu\text{g}/\text{ml}$  final concentration) were mixed 1:1 with 2X <sup>125</sup>I-c7E3 Fab (1  $\mu\text{g}/\text{ml}$  final concentration) and added to plates. The plates were incubated for 2 hours at 37°C. Plates were washed, wells separated into tubes, and bound radioactivity counted in a gamma counter. Data were presented as percent of maximal <sup>125</sup>I-c7E3 Fab bound in the absence of c7E3 Fab'-(PEG<sub>20,000</sub>)<sub>2</sub> or c7E3 Fab, and nonlinear regression analysis performed using GraphPad Prism.

Figures 3 and 4 show the data obtained for both c7E3 Fab and c7E3 Fab'-(PEG<sub>20,000</sub>)<sub>2</sub> with GPIIb/IIIa and  $\alpha_v\beta_3$  respectively, and indicate that the derivatization techniques used to create c7E3 Fab'-(PEG<sub>20,000</sub>)<sub>2</sub> do not have a significant effect on its binding.

Example VI Competition binding of c7E3 Fab'-(PEG<sub>5000</sub>)<sub>2</sub> with <sup>125</sup>I-c7E3 Fab to purified integrins

Purified  $\alpha_v\beta_3$  (Smith *et al.*, *J. Biol. Chem.* 263(35):18726-18731 (1988)) or GPIIb/IIIa (Pytela *et al.*, *Science* 231(4745):1559-1562 (1986)) was diluted in Tris-buffered saline (TBS) containing 2 mM calcium chloride (TBS/Ca) to a concentration of 0.5  $\mu\text{g}/\text{ml}$  ( $\alpha_v\beta_3$ ) or 1.5  $\mu\text{g}/\text{ml}$  (GPIIb/IIIa). 96 well polystyrene Immulon Removawell plates (Dynex Technologies, Chantilly, VA) were coated with  $\alpha_v\beta_3$  or GPIIb/IIIa by aliquoting 60  $\mu\text{l}$  of the appropriate protein solution into each well and incubating the plates at 4°C overnight. Plates were washed and blocked with 1% bovine serum albumin (BSA) in TBS/Ca for 1 hour at room temperature. Chimeric 7E3 Fab (c7E3 Fab) (ReoPro®, Centocor, Inc., Malvern, PA) was iodinated using Iodobeads® (Pierce Chemicals, Rockford, IL) to approximately 2  $\mu\text{Ci}/\mu\text{g}$ . Dilution series of unlabeled c7E3 Fab'-(PEG<sub>5000</sub>)<sub>2</sub> and unlabeled c7E3 Fab were prepared in TBS/Ca at 2X final concentration. Dilutions of c7E3 Fab'-(PEG<sub>5000</sub>)<sub>2</sub> or c7E3 Fab (starting at 20  $\mu\text{g}/\text{ml}$  final concentration) were mixed 1:1 with 2X <sup>125</sup>I-c7E3 Fab (1  $\mu\text{g}/\text{ml}$  final concentration) and added to plates. The plates were incubated for 2 hours at 37°C. Plates were washed, wells separated into tubes, and bound radioactivity counted in a gamma counter. Data were presented as



percent of maximal  $^{125}\text{I}$ -c7E3 Fab bound in the absence of c7E3 Fab'-(PEG<sub>5,000</sub>)<sub>2</sub> or c7E3 Fab, and nonlinear regression analysis performed using GraphPad Prism. Figures 5 and 6 show the data obtained for both c7E3 Fab and c7E3 Fab'-(PEG<sub>5,000</sub>)<sub>2</sub> with  $\alpha_v\beta_3$  and GPIIb/IIIa, respectively, and indicate that the derivatization techniques used to create c7E3 Fab'-(PEG<sub>5,000</sub>)<sub>2</sub> do not adversely affect the binding.

Example VII Competition binding of c7E3 Fab-PEG<sub>5000</sub> with  $^{125}\text{I}$ -c7E3 Fab to purified integrins

Purified  $\alpha_v\beta_3$  (Smith *et al.*, *J. Biol. Chem.* 263(35):18726-18731 (1988)) or GPIIb/IIIa (Pytela *et al.*, *Science* 231(4745):1559-1562 (1986)) was diluted in Tris-buffered saline (TBS) containing 2 mM calcium chloride (TBS/Ca) to 0.5  $\mu\text{g/ml}$  ( $\alpha_v\beta_3$ ) or 1.5  $\mu\text{g/ml}$  (GPIIb/IIIa). 96 well polystyrene Immulon Removawell plates (Dynex Technologies, Chantilly, VA) were coated with  $\alpha_v\beta_3$  or GPIIb/IIIa by aliquoting 60  $\mu\text{l}$  of the appropriate protein solution into each well and incubating the plates at 4°C overnight. Plates were washed, and blocked with 1% bovine serum albumin (BSA) in TBS/Ca for 1 hour at room temperature. Chimeric 7E3 Fab (c7E3 Fab, ReoPro®, Centocor, Inc., malvern, PA) was iodinated using Iodobeads® (Pierce Chemicals, Rockford, IL) to approximately 2  $\mu\text{Ci}/\mu\text{g}$ . Dilution series of unlabeled c7E3 Fab-PEG<sub>5000</sub> and unlabeled c7E3 Fab were prepared in TBS/Ca at 2X final concentration. Dilutions of c7E3 Fab-PEG<sub>5,000</sub> or c7E3 Fab (starting at 20  $\mu\text{g/ml}$  final concentration) were mixed 1:1 with 2X  $^{125}\text{I}$ -c7E3 Fab (1  $\mu\text{g/ml}$  final concentration) and added to plates. The plates were incubated for 2 hours at 37°C. Plates were washed, wells separated into tubes, and bound radioactivity counted in a gamma counter. Data were presented as percent of maximal  $^{125}\text{I}$ -c7E3 Fab bound in the absence of c7E3 Fab-PEG<sub>5,000</sub> or c7E3 Fab, and nonlinear regression analysis performed using GraphPad Prism.

Figures 7 and 8 show the data obtained for both c7E3 Fab and c7E3 Fab-PEG<sub>5,000</sub> for GPIIb/IIIa and  $\alpha_v\beta_3$ , respectively, and indicate that the derivatization techniques used to create c7E3 Fab-PEG<sub>5,000</sub> do not adversely affect the binding.

Example VIII GPIIb/IIIa Receptor Blockade (Fibrinogen Bead Assay)

GPIIb/IIIa complexes on activated platelets are receptors for fibrinogen. The mechanism of ligand binding is primarily by Arg-Gly-Asp (RGD) dependent process, whereby peptides containing the RGD sequence block platelet aggregation and inhibit the binding of fibrinogen. It was observed by Coller, *et al.* that platelets will agglutinate fibrinogen-coated beads via the GPIIb/IIIa fibrinogen interaction. Monoclonal antibodies that block the GPIIb/IIIa receptor can prevent the interaction of receptor and ligand and, thus, prevent agglutination of the fibrinogen coated beads.

10 Covalent Coupling of Fibrinogen to Beads (Carboxylated Polystyrene Microparticles)

Fibrinogen (Enzyme Research, South Bend, IN) was prewarmed to 37°C and reconstituted in warm water. Particulates were removed by centrifuge at 13,000-14,000 g for 7 minutes. Supernatant was removed and its absorbance at OD<sub>280</sub>, E=1.5 was measured. The fibrinogen solution was kept at room temperature until ready for use. The beads (Blue carboxylated polystyrene microparticles 3 µm diameter, 2.5% solids) were prepared as described (Coller *et al.*, *Circulation* 95(4):860-867 (1997)). Briefly, the beads were brought to room temperature and vortexed vigorously. Beads (5 ml) were placed into a 15 ml centrifuge tube with 0.1 M carbonate buffer, pH 9.6 and centrifuged for 10 minutes at room temp. The pellet was washed once again with carbonate buffer, centrifuged, and the supernatant discarded. The pellet was resuspended in 0.02 M Sodium phosphate buffer, pH 4.5, vortexed vigorously, and centrifuged for 10 minutes at ambient temperature. The supernatant was discarded and the pellet washed two additional times in phosphate buffer. Activation of the beads was accomplished by resuspending the pellet in 6.25 ml of phosphate buffer and slowly adding 6.25 ml of freshly made 2% carbodiimide solution. The bead/carbodiimide solution was mixed for 3 hours on a rocking platform at ambient temperature and then centrifuged for 10 minutes. The supernatant was removed and the pellet was washed three times in phosphate buffer to remove any unreacted carbodiimide. Once the removal of unreacted carbodiimide was complete, the supernatant was removed. At this point the pellet was

approximately 250  $\mu$ l. The pellet was resuspended in borate buffer to produce a 2% slurry. The 2% bead slurry was cooled in an ice bath with intermittent removal for vortexing to achieve monodispersity. The volume of fibrinogen stock needed to add 0.8 mg fibrinogen per ml of the original 2.5% slurry was calculated. The activated  
5 beads were diluted with borate buffer to a concentration that yielded a final reaction density of 1% solids slurry after fibrinogen was added. The beads were coated with fibrinogen overnight with gentle rocking at room temperature. The next day 0.1 ml of 0.25 M ethanolamine per ml of original 2.5% bead slurry was added. The solution was gently mixed for 30 minutes at room temperature to block unreacted  
10 sites on the beads. The bead solution was centrifuged for 10 minutes at room temperature. The supernatant was saved for protein determination. To ascertain how well the beads were coated, the amount of fibrinogen that was added originally was subtracted from the amount that was present in the supernatant. The bead pellet was resuspended to a 1% slurry with 10 mg/ml BSA in borate buffer. Again  
15 monodispersity was confirmed. The bead/BSA solution was mixed for 30 minutes at room temperature with gentle rocking to block any remaining nonspecific protein binding sites and to wash off loosely bound fibrinogen. The bead/BSA solution was centrifuged for 10 minutes at room temperature. The BSA blocking and centrifugation steps were repeated. The pellet was resuspended to a 2.5% slurry  
20 with storage buffer (PBS/1% BSA/0.1% sodium azide/5% glycerol). At this point the beads were stored at 4°C or used in a Fibrinogen Bead Assay.

#### Assay Setup

70  $\mu$ l of blood (1/100 sodium citrate from a human donor having received no medicine for 7 days) and 50  $\mu$ l antibody at varying concentrations (diluted in room  
25 temperature 10 mM TRIS/0.1% BSA), were mixed and incubated for 10 minutes at room temperature. The blood/antibody mixture was added to a glass test tube 12 x 75 mm containing 20  $\mu$ l fibrinogen-coated working beads, 105  $\mu$ l 10 mM HEPES buffer, and 20  $\mu$ M Thrombin Receptor Activating Peptide (Coller *et al.*,  
30 *Circulation* 95(4):860-867 (1997)). The tube was capped and placed on a rocking platform at speed 8 for 4 minutes. Agglutination of beads appeared as moderately sized blue clumps in the blood. As mentioned above, agglutination means that the

GPIIb/IIIa receptors are not blocked by the antibody tested.

Fibrinogen Bead Assay with c7E3 Fab'(PEG<sub>5,000</sub>)<sub>2</sub> compared to c7E3 Fab

The antibodies were tested in 2-fold dilutions starting at 50 µg/ml to 0.5 µg/ml according to the Assay Setup as described above. c7E3 Fab'(PEG<sub>5,000</sub>)<sub>2</sub> blocked bead agglutination at concentrations from 108-868 nM while c7E3 Fab (ReoPro®, Centocor, Inc., Malvern, PA) blocked from 131-1050 nM.

Fibrinogen Bead Assay with c7E3 Fab'(PEG<sub>20,000</sub>)<sub>2</sub> compared to c7E3 Fab

The antibodies were tested in 2-fold dilutions (from 50 µg/ml to 0.5 µg/ml) according to the Assay Setup as described above. c7E3 Fab'(PEG<sub>20,000</sub>)<sub>2</sub> blocks GPIIb/IIIa receptors from 143-571 nM while c7E3 Fab (ReoPro®, Centocor, Inc., Malvern, PA) blocks from 263-1050 nM.

Example IX Pharmacokinetics of c7E3 Fab'(PEG<sub>5,000</sub>)<sub>2</sub> in mice

On day 0 the study animals (CD-1 female mice weighing 25-30 g) were assigned to one of four treatment groups as shown in Table 1.

Table 1

| Group | N | Test Article                                       | Dose (mg/kg) | Route of admin. | Conc (mg/ml) | Dose Volume (ml/kg) | Post-dose Sample Time Points  |
|-------|---|--|--------------|-----------------|--------------|---------------------|-------------------------------|
| 1     | 3 | c7E3 Fab'-<br>(NEM) <sub>2</sub>                   | 10           | i.v.            | 1.5          | 6.7                 | 0.5, 2, 6 hours               |
| 2     | 3 | c7E3 Fab'-<br>(NEM) <sub>2</sub>                   | 10           | i.v.            | 1.5          | 6.7                 | 1, 4, 8 hours                 |
| 3     | 3 | c7E3 Fab'-<br>(PEG <sub>5,000</sub> ) <sub>2</sub> | 10           | i.v.            | 1.5          | 6.7                 | 6 hours, 2 days, 3 days       |
| 4     | 3 | c7E3 Fab'-<br>(PEG <sub>5,000</sub> ) <sub>2</sub> | 10           | i.v.            | 1.5          | 6.7                 | 1 hour, 1 day, 4 days, 5 days |

All animals were bled at designated time points. For all groups, 250  $\mu$ l of blood for all sample time points (except the final sample) was collected by retro-orbital sinus bleed and the terminal samples were taken by cardiac puncture. Blood was allowed to stand at room temperature for 30-60 minutes, centrifuged at 2500 rpm for 15 minutes and the serum separated and stored at -70°C until pharmacokinetic assays were performed.

#### Antibody Assay in Mouse Serum

C295A (a Centocor murine monoclonal anti-7E3-idiotypic antibody) was diluted in PBS to 5  $\mu$ g/ml and coated onto plates (Sterile, 96-well ELISA plates, Corning catalog #25805-96) using 50  $\mu$ l/well, and was incubated overnight at 4°C. Plates were then washed 3 times with wash buffer (0.9% sodium chloride, 0.02% Tween-20) using approximately 200  $\mu$ l/well per wash. Plates were blocked with a blocking buffer/sample diluent (1% BSA in PBS with 0.1% 2-chloroacetamide) for

1 hour at 37°C, then stored sealed at 4°C and warmed to room temperature before use. Standards were made from 50 µg/ml stocks which were made from the actual test articles (either c7E3 Fab'-NEM<sub>2</sub>, or c7E3 Fab'-(PEG<sub>5,000</sub>)<sub>2</sub>) and using the article which directly corresponded to that used in the animal test group (NEM = N-ethylmaleimide). The standard curve ranged from 1000 ng/ml to 0.95 ng/ml (in 1:2 serial dilutions). Standards were diluted in blocking buffer/sample diluent. Animal serum samples were also diluted in blocking buffer/sample diluent. Five 1:2 serial dilutions of each sample were made with the starting dilution being determined by estimation based on dose and time point. A plate blank of blocking buffer/sample diluent was added to each plate in triplicate at 50 µl/well. Standards and samples were applied to the plates in triplicate at 50 µl/well and incubated at 37°C for 1 hour. Plates were then washed as above. Test article captured from the standards/samples by the plated C295A monoclonal antibody was then detected by adding biotinylated C281A monoclonal antibody (a Centocor murine monoclonal anti-7E3 idiotype antibody that does not compete with C295A) diluted in blocking buffer/sample diluent at 50 µl/well. Plates were incubated at 37°C for 1 hour and then washed as above. Streptavidin-labelled horseradish peroxidase (Amersham, Catalog #RPN.1051) was diluted in blocking buffer/sample diluent and added to the plates at 50 µl/well and plates were incubated at 37°C for 30 minutes. Ten minutes prior to the end of the incubation step, OPD (o-phenylenediamine) tablets (Sigma, Catalog #P-8412) were dissolved in the OPD diluent (citrate/phosphate buffer with 0.03% hydrogen peroxide and 0.1% 2-chloroacetamide) to 1 mg/ml. Plates were then washed as above and the OPD substrate was added at 200 µl/well and allowed to develop, in the dark, at room temperature for 15 minutes. Development was stopped by the addition of 4 N H<sub>2</sub>SO<sub>4</sub> at 50 µl/well. The OD<sub>490</sub> was read for each plate and samples evaluated under a four parameter fit of the standards with the plate blank value being subtracted from all wells. The two dilutions closest to the midpoint of the standard curve were averaged for each sample dilution set. Samples which did not dilute out were repeated using either a higher, or lower, initial dilution as appropriate.

Other anti-7E3-idiotype antibodies suitable for use in the described assay can be prepared by known means. For example, a suitable host animal (e.g., mouse)

can be immunized with 7E3 and hybridomas can be prepared and screen by any suitable technique, such as those described herein.

Figure 9 shows the serum levels versus time of the test articles and demonstrates the significantly increased half-life of the c7E3 Fab'-(PEG<sub>5,000</sub>)<sub>2</sub> over the NEM-blocked Fab'.

#### Example X Pharmacokinetics of c7E3 Fab'-(PEG<sub>20,000</sub>)<sub>2</sub> in mice

On day 0 the study animals (CD-1 female mice weighing 25-30 g) were assigned to one of two treatment groups as shown in Table 2.

Table 2

| Group | n | Test Article                                    | Dose (mg/kg) | Route of admin. | Conc (mg/ml) | Dose Volume (ml/kg) | Post-dose Sample Time Points           |
|-------|---|---|--------------|-----------------|--------------|---------------------|--|
| 1     | 3 | C7E3 Fab'-(PEG <sub>20,000</sub> ) <sub>2</sub> | 18           | i.v.            | 1.3          | 13.8                | 6 hours,<br>2 days,<br>3 days          |
| 2     | 3 | C7E3 Fab'-(PEG <sub>20,000</sub> ) <sub>2</sub> | 18           | i.v.            | 1.3          | 13.8                | 1 hour,<br>1 day,<br>4 days,<br>5 days |

All animals were bled at designated time points. For all groups, 250 µL of blood for all sample time points (except the final sample) was collected by retro-orbital sinus and the terminal samples were taken by cardiac puncture. Blood was allowed to stand at room temperature for 30-60 minutes, centrifuged at 2500 rpm for 15 minutes and the serum separated and stored at -70°C until pharmacokinetic assays were performed. Assay for serum levels of c7E3 Fab'-(PEG<sub>20,000</sub>)<sub>2</sub> were performed as in Example IX.

Figure 10 shows the increased persistence in the serum of c7E3 Fab'-(PEG<sub>20,000</sub>)<sub>2</sub>.

Example XI Pharmacokinetics of c7E3 Fab'(PEG<sub>5,000</sub>)<sub>2</sub> in rats

Male CD1 rats weighing approximately 350 g were assigned to one of four treatment groups as shown in the Table 3.

Table 3

| Group | N | Test Article                                   | Dose (mg/kg) | Route of Admin. | Test Article (mg/ml) | Dose Volume (ml/kg) | Sample Time Points |
|-------|---|--|--------------|-----------------|----------------------|---------------------|--------------------|
| 1     | 3 | c7E3 Fab                                       | 20           | i.v.            | 2.0                  | 10                  | 1, 10, 60 min      |
| 2     | 3 | c7E3 Fab' (PEG <sub>5,000</sub> ) <sub>2</sub> | 20           | i.v.            | 2.0                  | 10                  | 0, 10, 60 min      |
| 3     | 3 | c7E3 Fab' (PEG <sub>5,000</sub> ) <sub>2</sub> | 20           | i.v.            | 2.0                  | 10                  | 6 hours            |
| 4     | 3 | Saline   | 20           | i.v.            | 0                    | 10                  | 6 hours            |

- 10 Animals were individually weighed and the test article dosages calculated. Indwelling intravenous catheters were inserted into the lateral tail veins and taped into place. Animals were anesthetized with sodium pentobarbital prior to the pre-dose blood sample and remained anesthetized throughout the entire course of blood sampling. Blood samples were collected according to the schedule in the above
- 15 table by cardiac puncture using 3 ml syringes containing 16  $\mu$ l of 40% sodium citrate. Blood samples were transferred from the syringes to 1.5 ml microfuge tubes and spun at 10,000 rpm briefly (4-5 seconds) in a microcentrifuge. The Platelet Rich Plasma (PRP) was removed from the top and the remainder of the blood sample spun at 10,000 rpm for 5 minutes in a microcentrifuge to obtain Platelet Poor
- 20 Plasma (PPP). Platelet counts of the PRP samples were determined and the platelet counts adjusted to between 200,000 and 300,000 platelets/ $\mu$ l with autologous PPP using a Coulter Counter ZM. The remaining PPP was frozen for determination of free Fab or Fab'(PEG<sub>5,000</sub>)<sub>2</sub> concentration.



Platelet aggregation was performed on a four-channel Bio/Data PAP4C aggregometer which allowed simultaneous evaluation of the aggregation responses of the pre-dose, 10 minute and 60 minute post-dose PRP samples for each animal. For each sample, 250  $\mu$ l of PRP was transferred to a siliconized aggregometer  
 5 cuvette and incubated without stirring at 37° C for 10 minutes. The baseline aggregation signal was monitored for 1 minute. ADP (10  $\mu$ M) was added to each cuvette and the aggregation response monitored for an additional 4 minutes. Maximal response (% light transmittance) for each PRP sample was recorded. The percent inhibition of the aggregation of the post-dose PRP samples relative to the  
 10 pre-dose PRP samples was determined using the following equation:

$$C = (A - B) / A \times 100$$

where:

C = % inhibition of aggregation of the PRP post-dose sample

A = % light transmittance of the PRP pre-dose sample

B = % light transmittance of the PRP post-dose sample

15 Serum levels of Fab or Fab'(PEG<sub>5,000</sub>)<sub>2</sub> were determined as outlined in Example VIII.

Figure 11 shows that the serum levels of Fab'(PEG<sub>5,000</sub>)<sub>2</sub> are more persistent than the corresponding Fab, showing conclusively the extended half-life of the Fab'(PEG<sub>5,000</sub>)<sub>2</sub>.

20 That the extended persistence of c7E3 Fab'(PEG<sub>5,000</sub>)<sub>2</sub> in serum is reflected in an increased duration of activity is shown by the ex-vivo aggregation data in the following table. Aggregation at 6 hours with c7E3 Fab'(PEG<sub>5,000</sub>)<sub>2</sub> is similar to aggregation at 60 minutes with c7E3 Fab.

Table 4

| Animal # | Test Article                                  | % aggregation |         |
|----------|---|---------------|---------|
|          |   | 60 min        | 6 hours |
| 1        | c7E3 Fab                                      | 14%           |         |
| 2        | c7E3 Fab                                      | 28%           |         |
| 3        | c7E3 Fab                                      | 38%           |         |
| 4        | c7E3 Fab'(PEG <sub>5,000</sub> ) <sub>2</sub> | 0%            |         |
| 5        | c7E3 Fab'(PEG <sub>5,000</sub> ) <sub>2</sub> | 0%            |         |
| 6        | c7E3 Fab'(PEG <sub>5,000</sub> ) <sub>2</sub> | 0%            |         |
| 7        | c7E3 Fab'(PEG <sub>5,000</sub> ) <sub>2</sub> |               | 18%     |
| 8        | c7E3 Fab'(PEG <sub>5,000</sub> ) <sub>2</sub> |               | 29%     |
| 9        | c7E3 Fab'(PEG <sub>5,000</sub> ) <sub>2</sub> |               | 26%     |

Example XII Determination of the binding kinetics of c7E3 Fab, c7E3 Fab', c7E3 F(ab')<sub>2</sub> or their analogs

A goat (Fab')<sub>2</sub> anti-human (Fab')<sub>2</sub> (Jackson Labs) was covalently immobilized to a BIAcore CM-5 (carboxymethyl) chip using NHS/EDC (N-hydroxysuccinimide/N-ethyl-N'-(dimethylaminopropyl) carbodiimide). The chip was activated with 40 µl of a 1:1 mixture of the coupling reagents obtained from BIAcore and prepared according to the manufacturers instructions at 10 µl/min. A 20 µg/ml solution of the (Fab')<sub>2</sub> in 10 mM sodium acetate, pH 4.8 was flowed over the chip at 10 µl/min until 2,500 RU were captured. Unreacted activated carboxyl groups were capped with a 40 µl injection of 1 M ethanolamine. Using a running buffer of 10 mM tris buffered saline, pH 7.5 containing 2 mM calcium chloride, 2 mM magnesium chloride, 0.01% Tween-100 and 25 µg/ml BSA, c7E3 Fab, c7E3 Fab' or c7E3 (Fab')<sub>2</sub> were flowed over the immobilized anti-human F(ab')<sub>2</sub> to capture 500-700 RU. 250 µl of a solution of GPIIb/IIIa or α<sub>v</sub>β<sub>3</sub> (from 2-10 µg/ml) were flowed over the capture antibody fragments at 30 µl/min followed by an

uninterrupted dissociation period of 800 seconds. The chip was regenerated with sequential 15  $\mu$ l injections of 100 mM phosphoric acid containing 0.05% CHAPS. The association and dissociation phases were analyzed using BIAevaluation 3.0 (BIAcore) for global association ( $k_a$ ) and dissociation ( $k_d$ ) constants.  $K_D$  was calculated by dividing  $k_a$  by  $k_d$ . The binding constants shown in Table 5 indicate that the derivatization techniques used to create the c7E3 Fab-PEG<sub>5,000</sub>, c7E3 Fab'-(PEG<sub>5,000</sub>)<sub>2</sub>, and c7E3 Fab'-(PEG<sub>20,000</sub>)<sub>2</sub> do not adversely affect the binding.

Table 5

| Binding constants.                              |  |                            |                       |
|---|--|----------------------------|-----------------------|
|   | $k_a$ (mol <sup>-1</sup> sec <sup>-1</sup> ) | $k_d$ (sec <sup>-1</sup> ) | $K_D$ (M)             |
| c7E3 Fab  | $4.55 \times 10^4$                           | $5.22 \times 10^{-5}$      | $1.15 \times 10^{-9}$ |
| c7E3 Fab'-(PEG <sub>5,000</sub> ) <sub>2</sub>  | $4.87 \times 10^4$                           | $8.15 \times 10^{-5}$      | $1.67 \times 10^{-9}$ |
| c7E3 Fab'-(PEG <sub>20,000</sub> ) <sub>2</sub> | $4.48 \times 10^4$                           | $10.60 \times 10^{-5}$     | $2.36 \times 10^{-9}$ |
| c7E3 Fab PEG <sub>5,000</sub>                   | $1.71 \times 10^4$                           | $7.65 \times 10^{-5}$      | $4.47 \times 10^{-9}$ |

Example XIII      Immunogenicity of c7E3 Fab'-(PEG<sub>5,000</sub>)<sub>2</sub> as compared to c7E3 Fab

#### Animals and Reagents:

Two groups of six week old Balb/c mice from Charles River Laboratories (eight animals per group) were immunized by intra peritoneal (IP) injection with 100  $\mu$ g c7E3 Fab (ReoPro<sup>®</sup>, Centocor, Inc., Malvern, PA) or c7E3 Fab'-(PEG<sub>5,000</sub>)<sub>2</sub> diluted in physiological saline (Baxter) and emulsified in an equal volume of complete Freund's adjuvant. A subsequent IP injection with 100  $\mu$ g c7E3 Fab (ReoPro<sup>®</sup>, Centocor, Inc., Malvern, PA) or c7E3 Fab'-(PEG<sub>5,000</sub>)<sub>2</sub> emulsified in incomplete Freund's adjuvant was given 14 days later. The mice were bled by retro-

orbital puncture without anti-coagulant on day 20. The blood was allowed to clot at room temperature for one hour and the serum was collected.

The immunogenicity of c7E3 Fab or c7E3 Fab'(PEG<sub>5,000</sub>)<sub>2</sub> was evaluated by measuring the immune response via a solid phase enzyme linked immunoassay

5 (EIA). c7E3 Fab was coated on 96-well flat bottom EIA plates at 10 µg/ml in 10 mM carbonate buffer, pH 9.6, 50 µL/well overnight at 4°C. After washing in 0.15 M saline and 0.02% (v/v) Tween 20, the wells were blocked with 1% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS), 200 µL/well for 1 hour at RT. Plates were used immediately or frozen at -20 C for future use.

10 All sera were diluted 1:50 in PBS and stored at 4°C until tested. Doubling dilutions of immune sera were incubated on the immunogen coated plates at 50 µL/well at room temperature (RT) for 1 hour. The plates were washed and then probed with 50 µL/well goat anti-mouse IgG, Fc specific, horseradish peroxidase labeled conjugate diluted 1:20,000 in 1% BSA-PBS for 1 hour at RT. The plates  
15 were again washed and 100 µL/well of the citrate-phosphate substrate solution [0.1M citric acid and 0.2M sodium phosphate, 0.01% H<sub>2</sub>O<sub>2</sub> (Sigma) and 1 mg/mL phenylenediamine dihydrochloride] was added for 15 minutes at RT. Stop solution (4 N sulfuric acid) was then added at 25 µL/well and the optical densities were read at 490 nm on a Dynatech MR 5000 automated plate spectrophotometer.

20 Figure 12 shows the inverse geometric mean of the titers. C7E3 Fab'(PEG<sub>5,000</sub>)<sub>2</sub> shows significantly less immunogenicity than c7E3 Fab.

#### Example XIV      Synthesis of N-Maleimidoacetyl-N'-palmitoyl-PEG<sub>3,400</sub>

##### Step 1

Palmitoyl chloride (80.8 mg, 0.2942 mM) was dissolved in 2 mL acetone  
25 and added dropwise to a solution of N-*tert*-butoxycarbonyl-PEG<sub>3,400</sub> diamine (Shearwater Polymers, Inc.)(500 mg, 0.1471 mM) in 5.0 mL of pyridine and the resulting reaction mixture was refluxed for three hours. The pyridine was evaporated under reduced pressure and the resulting oil was treated with 40 mL of ethyl ether to give a white precipitate that was removed by filtration, washed with  
30 ether and dried under reduced pressure to give 554 mg of white solid. The crude material was purified using silica gel column chromatography (Baker Silica Gel, 40

μm, flash chromatography packing, glass column, 2 x 30 cm) eluting with chloroform : methanol (9:1, v/v). Fractions were pooled, evaporated to dryness and triturated with ethyl ether. A white precipitate was recovered by filtration, washed with ether then dried under reduced pressure to give 266 mg of N-*tert*-butoxycarbonyl-N'-palmitoyl-PEG<sub>3,400</sub> (MH<sup>+</sup> 3770.15 (cluster)).

#### Step 2

N-*tert*-butoxycarbonyl-N'-palmitoyl-PEG<sub>3,400</sub> (227 mg) was stirred with 25 mL of trifluoroacetic acid : dichloromethane (1:1, v/v) for 15 minutes at ambient temperature. Solvents were evaporated under reduced pressure and ethyl ether (100 mL) was added to the residue. The resulting white precipitate was recovered by filtration, washed with ether and dried under reduced pressure to yield 141 mg of N-palmitoyl-PEG<sub>3,400</sub> amine trifluoroacetate.

#### Step 3

N-palmitoyl-PEG<sub>3,400</sub> amine trifluoroacetate (350 mg, 0.0925 mM) was dissolved in 3.5 mL tetrahydrofuran and diisopropylethylamine (40 mL) was added to adjust the pH to about 8.0 (as measured with a moistened indicator paper). After about 10 minutes of stirring, maleimido acetic acid N-hydroxysuccinamide ester (48 mg, 0.185 mM) (Molecular Biosciences, Lot 11158) was added and the mixture was stirred for about an additional four hours. The addition of diethyl ether (90 mL) resulted in the precipitation of a white solid that was isolated and dried under reduced pressure to yield 330 mg of N-Maleimidoacetyl-N'-palmitoyl-PEG<sub>3,400</sub> (MH<sup>+</sup> 3806.89 (cluster)).

Example XV      Synthesis of c7E3 Fab'(Palmitoyl-PEG<sub>3,400</sub>)<sub>2</sub>

c7E3 F(ab)<sub>2</sub>' (43.6 mg in 9 mL of PBS) was placed in a water bath (37°C). 135 µL of 1.0 M L-cysteine (Sigma, St. Louis) in water was added and the reaction mixture let stand at 30°C for 30 minutes. Ammonium sulfate (714 mg) was added and the reaction mixture was loaded onto an HPLC column (Tosohaas, Phenyl 5PW, 10 µm, 0.75 x 7.5 cm) and eluted with a 60 minute linear gradient (0-100%) of 0.1 M sodium phosphate/0.6 M ammonium sulfate/0.05% EDTA, pH 6.5 to 0.1 M sodium phosphate/0.05% EDTA, pH 6.5 at a flow rate of 6 mL per minute. The peak corresponding to c7E3 Fab' was collected. To this was added 28 mg of N-Maleimidoacetyl-N'-palmitoyl-PEG<sub>3,400</sub> and the reaction was gently agitated for about 30 minutes to give the titled compound (MH<sup>+</sup> 56,362.4 (cluster)).

Example XVI      Synthesis of 7E3 Fab'(PEG<sub>10,000</sub>)<sub>2</sub>

c7E3 F(ab)<sub>2</sub>' (28.8 mg in 9.5 mL of PBS) was placed in a water bath (37°C). 182 µL of 1.0 M L-cysteine (Sigma, St. Louis) in water was added and the reaction mixture let stand at 37°C for 30 minutes. Ammonium sulfate (761 mg) was added and the reaction mixture was loaded onto an HPLC column (Tosohaas, Phenyl 5PW, 10 µm, 0.75 x 7.5 cm) and eluted with a 60 minute linear gradient (0-100%) of 0.1 M sodium phosphate/0.6 M ammonium sulfate/0.05% EDTA, pH 6.5 to 0.1 M sodium phosphate/0.05% EDTA, pH 6.5 at a flow rate of 6 mL per minute. The peak corresponding to c7E3 Fab' was collected. Methoxy-PEG<sub>10,000</sub>-maleimide (Shearwater polymers, Inc) (46.5 mg) was added to the Fab' solution and gently agitated for 15 hours at ambient temperature. The reaction mixture was concentrated to 10 mL and loaded onto an HPLC column (Tosohaas, Phenyl 5PW, 10 µm, 0.75 x 7.5 cm) and eluted with a 60 minute linear gradient (0-100%) of 0.1 M sodium phosphate/0.6 M ammonium sulfate, pH 7.5 to 0.1 M sodium phosphate, pH 7.5 at a flow rate of 6 mL per minute. The peak corresponding to the title compound was collected, concentrated and reloaded onto an HPLC column (Tosohaas, Phenyl 5PW, 10 µm, 0.75 x 7.5 cm) and eluted with a 60 minute linear gradient (50-70%) of 0.1 M sodium phosphate/0.6 M ammonium sulfate, pH 7.5 to 0.1 M sodium phosphate, pH 7.5 at a flow rate of 6 mL per minute. The peak

correspond to the titled compound was collected and concentrated to yield 9 mg of product ( $MH^+$  69,832 (cluster)).

Example XVII      Synthesis of c7E3Fab'( $PEG_{2,000}$ )<sub>4</sub>)<sub>2</sub>

- c7E3 F(ab)'<sub>2</sub> (40.1 mg in 9.5 mL of PBS) was placed in a water bath (37°C).
- 5    163  $\mu$ L of 1.0 M L-cysteine (Sigma, St. Louis) in water was added and the reaction mixture let stand at 37°C for 30 minutes. Ammonium sulfate (777 mg) was added and the reaction mixture was loaded onto an HPLC column (Tosohaas, Phenyl 5PW, 10  $\mu$ m, 0.75 x 7.5 cm) and eluted with a 60 minute linear gradient (0-100%) of 0.1 M sodium phosphate/0.6 M ammonium sulfate/0.05% EDTA, pH 6.5 to 0.1 M
- 10   sodium phosphate/0.05% EDTA, pH 6.5 at a flow rate of 6 mL per minute. The peak corresponding to c7E3 Fab' was collected. ( $PEG_{2,000}$ )<sub>4</sub>-maleimide (Shearwater Polymers, Inc.) (50.2 mg) was added to the Fab' solution and gently agitated for 2 hours at ambient temperature. The reaction mixture was concentrated to 9.8 mL and loaded onto an HPLC column (Tosohaas, Phenyl 5PW, 10  $\mu$ m, 0.75 x 7.5 cm) and
- 15   eluted with a 60 minute linear gradient (0-100%) of 0.1 M sodium phosphate/0.6 M ammonium sulfate, pH 7.5 to 0.1 M sodium phosphate, pH 7.5 at a flow rate of 6 mL per minute. The peak corresponding to the title compound was collected, concentrated and reloaded onto an HPLC column (Tosohaas, Phenyl 5PW, 10  $\mu$ m, 0.75 x 7.5 cm) and eluted with a 60 minute linear gradient (50-70%) of 0.1 M
- 20   sodium phosphate/0.6 M ammonium sulfate, pH 7.5 to 0.1 M sodium phosphate, pH 7.5 at a flow rate of 6 mL per minute. The peak correspond to the titled compound was collected and concentrated to yield 13.5 mg of product ( $MH^+$  65,942 (cluster)).

Example XVIII      Synthesis of c7E3Fab'(( $PEG_{2,000}$ )<sub>2</sub>)<sub>2</sub>

- 25      c7E3 F(ab)'<sub>2</sub> (54.3 mg in 10 mL of PBS) was placed in a water bath (37°C). 342  $\mu$ L of 1.0 M L-cysteine (Sigma, St. Louis) in water was added and the reaction mixture let stand at 37°C for 30 minutes. Ammonium sulfate (756 mg) was added and the reaction mixture was loaded onto an HPLC column (Tosohaas, Phenyl 5PW, 10  $\mu$ m, 0.75 x 7.5 cm) and eluted with a 60 minute linear gradient (0-100%) of 0.1
- 30   M sodium phosphate/0.6 M ammonium sulfate/0.05% EDTA, pH 6.5 to 0.1 M

sodium phosphate/0.05% EDTA, pH 6.5 at a flow rate of 6 mL per minute. The peak corresponding to c7E3 Fab' was collected. (PEG<sub>2,000</sub>)<sub>2</sub>-maleimide (Shearwater Polymers, Inc.) (39.3 mg) was added to the Fab' solution and gently agitated for 2.5 hours at ambient temperature. The reaction mixture was concentrated to 9.8 mL and  
5 loaded onto an HPLC column (Tosohaas, Phenyl 5PW, 10 µm, 0.75 x 7.5 cm) and eluted with a 60 minute linear gradient (0-100%) of 0.1 M sodium phosphate/0.6 M ammonium sulfate, pH 7.5 to 0.1 M sodium phosphate, pH 7.5 at a flow rate of 6 mL per minute. The peak corresponding to the title compound was collected, concentrated and reloaded onto an HPLC column (Tosohaas, Phenyl 5PW, 10 µm,  
10 0.75 x 7.5 cm) and eluted with a 60 minute linear gradient (50-70%) of 0.1 M sodium phosphate/0.6 M ammonium sulfate, pH 7.5 to 0.1 M sodium phosphate, pH 7.5 at a flow rate of 6 mL per minute. The peak correspond to the titled compound was collected and concentrated to yield 15.6 mg of product (MH<sup>+</sup> 57,313.5 (cluster)).

15 Example XIX      Synthesis of c7E3Fab'((PEG<sub>5,000</sub>)<sub>2</sub>)<sub>2</sub>  
c7E3 F(ab)'<sub>2</sub> (54.3 mg in 10mL of PBS) was placed in a water bath (37°C). 342 µL of 1.0 M L-cysteine (Sigma, St. Louis) in water was added and the reaction mixture let stand at 37°C for 30 minutes. Ammonium sulfate (756 mg) was added and the reaction mixture was loaded onto an HPLC column (Tosohaas, Phenyl 5PW,  
20 10 µm, 0.75 x 7.5 cm) and eluted with a 60 minute linear gradient (0-100%) of 0.1 M sodium phosphate/0.6 M ammonium sulfate/0.05% EDTA, pH 6.5 to 0.1 M sodium phosphate/0.05% EDTA, pH 6.5 at a flow rate of 6 mL per minute. The peak corresponding to c7E3 Fab' was collected. (PEG<sub>5,000</sub>)<sub>2</sub>-maleimide (Shearwater Polymers, Inc.) (104 mg) was added to the Fab' solution and gently agitated for 2.5  
25 hours at ambient temperature. The reaction mixture was concentrated to 9.8 mL and loaded onto an HPLC column (Tosohaas, Phenyl 5PW, 10 µm, 0.75 x 7.5 cm) and eluted with a 60 minute linear gradient (0-100%) of 0.1 M sodium phosphate/0.6 M ammonium sulfate, pH 7.5 to 0.1 M sodium phosphate, pH 7.5 at a flow rate of 6 mL per minute. The peak corresponding to the title compound was collected,  
30 concentrated and reloaded onto an HPLC column (Tosohaas, Phenyl 5PW, 10 µm, 0.75 x 7.5 cm) and eluted with a 60 minute linear gradient (50-70%) of 0.1 M



sodium phosphate/0.6 M ammonium sulfate, pH 7.5 to 0.1 M sodium phosphate, pH 7.5 at a flow rate of 6 mL per minute. The peak correspond to the titled compound was collected and concentrated to yield 14.2 mg of product ( $MH^+$  71,194(cluster)).

5 Example XX Synthesis of c7E3Fab'(PEG<sub>2,000</sub>)<sub>2</sub>

c7E3 F(ab)'<sub>2</sub> (40.1 mg in 9.5 mL of PBS) was placed in a water bath (37°C). 163 µL of 1.0 M L-cysteine (Sigma, St. Louis) in water was added and the reaction mixture let stand at 37°C for 30 minutes. Ammonium sulfate (777 mg) was added and the reaction mixture was loaded onto an HPLC column (Tosohaas, Phenyl 5PW, 10 µm, 10 0.75 x 7.5 cm) and eluted with a 60 minute linear gradient (0-100%) of 0.1 M sodium phosphate/0.6 M ammonium sulfate/0.05% EDTA, pH 6.5 to 0.1 M sodium phosphate/0.05% EDTA, pH 6.5 at a flow rate of 6 mL per minute. The peak corresponding to c7E3 Fab' was collected. PEG<sub>2,000</sub>-maleimide (Shearwater Polymers, Inc.) (20 mg) was added to the Fab' solution and gently agitated for 2.5 hours at 15 ambient temperature. The reaction mixture was concentrated to 9.8 mL and loaded onto an HPLC column (Tosohaas, Phenyl 5PW, 10 µm, 0.75 x 7.5 cm) and eluted with a 60 minute linear gradient (0-100%) of 0.1 M sodium phosphate/0.6 M ammonium sulfate, pH 7.5 to 0.1 M sodium phosphate, pH 7.5 at a flow rate of 6 mL per minute. The peak corresponding to the title compound was collected, concentrated and reloaded onto an 20 HPLC column (Tosohaas, Phenyl 5PW, 10 µm, 0.75 x 7.5 cm) and eluted with a 60 minute linear gradient (50-70%) of 0.1 M sodium phosphate/0.6 M ammonium sulfate, pH 7.5 to 0.1 M sodium phosphate, pH 7.5 at a flow rate of 6 mL per minute. The peak correspond to the titled compound was collected and concentrated to yield 10.4 mg of product ( $MH^+$  52,817 (cluster)).

25 Example XXI Inhibition of Platelet Aggregation

c7E3, c7E3 Fab'(PEG<sub>2,000</sub>)<sub>2</sub>, c7E3 Fab'((PEG<sub>2,000</sub>)<sub>2</sub>)<sub>2</sub>, c7E3 Fab'((PEG<sub>2,000</sub>)<sub>4</sub>)<sub>2</sub> and c7E3 Fab'((PEG<sub>5,000</sub>)<sub>2</sub>)<sub>2</sub> were tested for platelet aggregation inhibiting activity as

described in Example IV. The results of these studies are presented graphically in Figures 13-15 and further demonstrate that chemical modification of c7E3 to increase half-life does not affect activity.

Example XXII      Determination of the binding kinetics of modified antibodies

- 5            Binding kinetics were measured and rate constants and affinities for binding to GPIIb/IIIa were calculated as described in Example XII. The rate constants and affinities reported in Tables 5 (Example XII) and 6 are the average of at least two and up to about seven independent determinations. The data used to calculate the rate constants and affinities reported in Table 5 were included with additional data in
- 10           calculating the rate constants and affinities reported in Table 6. The rate constants and affinities reported in Table 6 further demonstrate that site specific modification of c7E3 can be accomplished without adversely affect binding, while random modification dramatically reduces affinity.

Table 6 Binding constants

|   | $k_a$ ( $\text{mol}^{-1}\text{sec}^{-1}$ ) | $k_d$ ( $\text{sec}^{-1}$ ) | $K_D$ (M)            |
|---|--|-----------------------------|----------------------|
| c7E3 Fab  | $2.9 \times 10^4$                          | $7.6 \times 10^{-5}$        | $3.8 \times 10^{-9}$ |
| c7E3 Fab (PEG <sub>5,000</sub> )                                  | $1.7 \times 10^4$                          | $7.7 \times 10^{-5}$        | $4.5 \times 10^{-9}$ |
| 5 c7E3 Fab (PEG <sub>20,000</sub> )                               | $1.2 \times 10^5$                          | $1.9 \times 10^{-4}$        | $1.1 \times 10^{-8}$ |
| c7E3 Fab' (PEG <sub>2,000</sub> ) <sub>2</sub>                    | $1.6 \times 10^4$                          | $6.8 \times 10^{-5}$        | $2.5 \times 10^{-9}$ |
| c7E3 Fab' (PEG <sub>5,000</sub> ) <sub>2</sub>                    | $3.8 \times 10^4$                          | $9.8 \times 10^{-5}$        | $3.0 \times 10^{-9}$ |
| c7E3 Fab' (PEG <sub>10,000</sub> ) <sub>2</sub>                   | $3.1 \times 10^4$                          | $9.6 \times 10^{-5}$        | $3.1 \times 10^{-9}$ |
| c7E3 Fab' (PEG <sub>20,000</sub> ) <sub>2</sub>                   | $2.4 \times 10^4$                          | $9.0 \times 10^{-5}$        | $4.9 \times 10^{-9}$ |
| 10 c7E3 Fab' ((PEG <sub>2,000</sub> ) <sub>2</sub> ) <sub>2</sub> | $2.6 \times 10^4$                          | $6.8 \times 10^{-5}$        | $2.9 \times 10^{-9}$ |
| c7E3 Fab' ((PEG <sub>5,000</sub> ) <sub>2</sub> ) <sub>2</sub>    | $1.4 \times 10^4$                          | $7.4 \times 10^{-5}$        | $5.6 \times 10^{-9}$ |
| c7E3 Fab Random (PEG <sub>5,000</sub> )                           | $8.2 \times 10^3$                          | $1.5 \times 10^{-5}$        | $1.9 \times 10^{-8}$ |

## Example XXIII

## Pharmacokinetics in mice

- 15 Pharmacokinetics were performed in mice using a protocol similar to those described in Examples IX and X. Mice were given an intravenous bolus of test article, blood was drawn at predetermined time points and the serum concentration of the test article was determined. Seven independent studies using 3-5 mice were conducted. A different test article was administered for each study, as follows: Study 1, c7E3 Fab was
- 20 administered at a dose of 20 mg/kg; Study 2, c7E3 Fab PEG<sub>5,000</sub> was administered at a dose of 20 mg/kg; Study 3, c7E3 Fab'((PEG<sub>2,000</sub>)<sub>2</sub>)<sub>2</sub> was administered at a dose of 0.6 mg/kg; Study 4, c7E3 Fab PEG<sub>20,000</sub> was administered at a dose of 0.65 mg/kg; Study 5, c7E3 Fab' (PEG<sub>2,000</sub>)<sub>2</sub> was administered at a dose of 0.56 mg/kg; Study 6, c7E3 Fab'((PEG<sub>5,000</sub>)<sub>2</sub>)<sub>2</sub> was administered at a dose of 0.75 mg/kg; Study 7, c7E3
- 25 Fab' (PEG<sub>10,000</sub>)<sub>2</sub> was administered at a dose of 0.74 mg/kg. The results of each study are presented in Table 7, and demonstrate that c7E3 Fab PEG<sub>5,000</sub>, c7E3 Fab'((PEG<sub>2,000</sub>)<sub>2</sub>)<sub>2</sub>, c7E3 Fab PEG<sub>20,000</sub>, c7E3 Fab' PEG((<sub>5,000</sub>)<sub>2</sub>)<sub>2</sub> and c7E3 Fab' PEG(<sub>10,000</sub>)<sub>2</sub> persist in the

serum longer than unmodified c7E3 Fab after intravenous administration. These studies also revealed that c7E3 Fab'(PEG<sub>2,000</sub>)<sub>2</sub> persists in the serum for about as long as unmodified c7E3 Fab.

Table 7 Pharmacokinetics in mice

| Time<br>(h) | Serum Concentration of Test Article (nM) |   |   |  |   |   |  |
|-------------|--|---|---|--|---|---|--|
|             | Study 1<br>(c7E3 Fab)                    | Study 2<br>(c7E3 Fab PEG <sub>5,000</sub> ) | Study 3<br>(c7E3 Fab'((PEG <sub>1,000</sub> ) <sub>2</sub> ) <sub>2</sub> ) | Study 4<br>(c7E3 Fab PEG <sub>10,000</sub> ) | Study 5<br>(c7E3 Fab'(PEG <sub>2,000</sub> ) <sub>2</sub> ) | Study 6<br>(c7E3 Fab'((PEG <sub>5,000</sub> ) <sub>2</sub> ) <sub>2</sub> ) | Study 7<br>(c7E3 Fab'(PEG <sub>10,000</sub> ) <sub>2</sub> ) |
| 0.17        | 5.7 <sup>1</sup>                         | 6.0   | nd  | nd   | nd  | nd  | nd   |
| 1           | 1.3                                      | 5.9   | 91.3  | 92.4   | 63.3  | 228.7   | 303.9  |
| 6           | nd                                       | 1.5   | 43.1  | 73.3   | 3.0   | 160.3   | 130.2  |
| 24          | nd                                       | nd  | 2.9   | 17.7   | 0.2   | 44.8  | 79.4   |
| 48          | nd                                       | nd  | 1.0   | 10.4   | 0.0   | 34.9  | 29.6   |
| 72          | nd                                       | nd  | 0.2   | 3.0  | nd  | 11.0  | 15.6   |
| 96          | nd                                       | nd  | 0.1   | nd   | nd  | 5.2   | 13.0   |

nd, not determined

Example XXIV Pharmacodynamics in cynomolgus monkeys

Seven independent studies using 2-4 cynomolgus monkeys were conducted. Each animal was instrumented with a vascular access port (VAPs) for obtaining blood samples. An initial blood sample was acquired and platelet aggregation was measured at the time of VAP placement. Each animal was administered a single bolus of test article by intravenous injection. A different test article was administered for each study, as follows: Study 1, c7E3 Fab was administered at a dose of 0.4 mg/kg; Study 2, c7E3 Fab'(PEG<sub>5,000</sub>)<sub>2</sub> was administered at a dose of 1.05 mg/kg; Study 3, c7E3 Fab'(PEG<sub>10,000</sub>)<sub>2</sub> was administered at a dose of 0.6 mg/kg; Study 4, c7E3 Fab'(PEG<sub>5,000</sub>)<sub>2</sub> was administered at a dose of 0.6 mg/kg; Study 5, c7E3 Fab'(PEG<sub>20,000</sub>)<sub>2</sub> was administered at a dose of 1.4 mg/kg; Study 6, c7E3 Fab'(PEG<sub>2,000</sub>)<sub>2</sub> was administered at a dose of 0.45 mg/kg. Blood was collected prior to administration of test article and at predetermined time points, for assessment of platelet function, hematology and pharmacokinetics. Platelet aggregation was measured as described in Example IV. Serum levels of test article were evaluated as described in Example VIII. The results of the studies are presented in Tables 8a and 8b.

The studies revealed that platelet aggregation was inhibited by 96% one hour after administration of c7E3 Fab, but that inhibition declined rapidly thereafter. The amount of c7E3 which was detected in the serum also rapidly declined after one hour, paralleling the inhibition of platelet aggregation. Similar results were obtained when c7E3 Fab'(PEG<sub>2,000</sub>)<sub>2</sub> was administered, demonstrating that modification with two linear PEG chains of molecular weight 2,000 did not increase serum persistence in comparison with the unmodified Fab. In contrast, the administration of Fab' fragments which were modified with PEG chains of molecular weight greater than 2,000 resulted in prolonged inhibition of platelet aggregation and a slower decline in inhibitory activity. Similarly, the quantity of Fab' fragments modified with PEG chains of molecular weight greater than 2,000 which were detected in serum one hour after administration was higher than for the unmodified Fab', and the serum levels declined at a slower rate.

Table 8a      Pharmacodynamics in monkeys

| Time<br>(hours) | Study 1<br>(c7E3 Fab)                    |                                | Study 2<br>(c7E3 Fab'(PEG <sub>5,000</sub> ) <sub>2</sub> ) |                                | Study 3<br>(c7E3 Fab'(PEG <sub>10,000</sub> ) <sub>2</sub> ) |                                |
|-----------------|--|--------------------------------|---|--------------------------------|--|--------------------------------|
|                 | platelet<br>aggregation<br>(%inhibition) | serum<br>concentration<br>(nM) | platelet<br>aggregation<br>(% inhibition)                   | serum<br>concentration<br>(nM) | platelet<br>aggregation<br>(% inhibition)                    | serum<br>concentration<br>(nM) |
| 0.17            | nd                                       | nd                             | nd  | nd                             | 88   | 58.9                           |
| 5 1             | 96                                       | 28.8                           | 100   | 285.0                          | 86   | 43.1                           |
| 6               | 29                                       | 1.9                            | 99  | 145.6                          | nd   | nd                             |
| 12              | 15                                       | 1.0                            | 77  | 88.3                           | 60   | 15.0                           |
| 24              | 5  | 0.5                            | 32  | 29.4                           | 28   | 8.1                            |
| 36              | 7  | 0.3                            | 23  | 15.6                           | 28   | 5.2                            |
| 10 48           | 1  | 0.2                            | 22  | 10.5                           | 26   | 3.0                            |

nd, not determined

Table 8b Pharmacodynamics in monkeys

| Time<br>(hours) | Study 4<br>(c7E3 Fab'((PEG <sub>5,000</sub> ) <sub>2</sub> ) <sub>2</sub> ) |                                | Study 5<br>(c7E3 Fab'(PEG <sub>20,000</sub> ) <sub>2</sub> ) |                                | Study 6<br>(c7E3 Fab'(PEG <sub>2,000</sub> ) <sub>2</sub> ) |                                |
|-----------------|---|--------------------------------|--|--------------------------------|---|--------------------------------|
|                 | platelet<br>aggregation<br>(%inhibition)                                    | serum<br>concentration<br>(nM) | platelet<br>aggregation<br>(% inhibition)                    | serum<br>concentration<br>(nM) | platelet<br>aggregation<br>(% inhibition)                   | serum<br>concentration<br>(nM) |
| 1               | 73  | 89.9                           | 100  | 185.4                          | 75  | 59.7                           |
| 5 4             | nd  | nd                             | 100  | 128.2                          | nd  | nd                             |
| 6               | 59  | 50.5                           | nd   | nd                             | 36  | 10.5                           |
| 8               | nd  | nd                             | 100  | 96.7                           | nd  | nd                             |
| 12              | 30  | 30.0                           | 100  | 80.4                           | 19  | 3.5                            |
| 24              | 11  | 19.7                           | 99   | 49.6                           | 15  | 1.8                            |
| 10 36           | 0   | 11.5                           | nd   | nd                             | 12  | 1.1                            |
| 48              | 0   | 7.5                            | 77   | 21.7                           | 17  | 0.8                            |
| 120             | nd  | nd                             | 7  | 3.9                            | nd  | nd                             |

nd, not determined

15 Example XXV c7E3 Fab-PEG<sub>20,000</sub>

The titled compound was prepared as described in Example III except substituting PEG<sub>20,000</sub> aldehyde for PEG<sub>5,000</sub> aldehyde.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that  
 20 various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.



## CLAIMS

What is claimed is:

1. A modified antibody or modified antigen-binding fragment that specifically  
5 binds to one or more antigens selected from the group consisting of GPIIb/IIIa,  $\alpha_v\beta_3$  and Mac-1, the modification comprising one or more organic moieties which are covalently bonded to said antibody or antigen-binding fragment.
2. The modified antibody or modified antigen-binding fragment of Claim 1  
10 wherein said modification comprises one to about six organic moieties, which are each, independently, a hydrophilic polymeric group, a fatty acid group, a fatty acid ester group, a lipid or a phospholipid.
3. The modified antibody or modified antigen-binding fragment of Claim 2  
15 wherein an organic moiety is covalently bonded to at least one carboxyl-terminus of said antibody or antigen-binding fragment.
4. The modified antigen-binding fragment of Claim 3 wherein said antigen-binding fragment is an Fab.
5. The modified antigen-binding fragment of Claim 4 wherein said Fab is an achromopeptidase fragment.
- 20 6. The modified antigen-binding fragment of Claim 3 wherein said organic moiety is bonded to the carboxyl-terminus of the heavy chain.

7. The modified antibody or modified antigen-binding fragment of Claim 3 further comprising one to about four additional organic moieties which are each independently covalently bonded to the side chain sulfur atom of a cysteinyl residue.
- 5 8. The modified antibody or modified antigen-binding fragment of Claim 7 wherein the heavy chain comprises said cysteinyl residue.
9. The modified antibody or modified antigen-binding fragment of Claim 2 wherein an organic moiety is covalently bonded to the side chain sulfur atom of at least one cysteinyl residue.
- 10 10. The modified antibody or modified antigen-binding fragment of Claim 9 wherein the heavy chain comprises said cysteinyl residue.
11. The modified antigen-binding fragment of Claim 9 wherein said antigen binding fragment is an Fab' and said organic moiety is bonded to a sulfur atom of at least one heavy chain cysteinyl residue that forms an inter-chain disulfide bond.
- 15 12. The modified antibody or modified antigen-binding fragment of Claim 2 that specifically binds GPIIb/IIIa, inhibits binding of fibrinogen to GPIIb/IIIa, and inhibits platelet aggregation.
13. The modified antibody or modified antigen-binding fragment of Claim 12  
20 wherein said antibody or antigen-binding fragment further specifically binds  $\alpha_v\beta_3$ .

14. The modified antibody or modified antigen-binding fragment of Claim 13 wherein the binding of said antibody or antigen-binding fragment to GPIIb/IIIa and/or  $\alpha_v\beta_3$  is competitively inhibited by 7E3.
- 5 15. The modified antibody or modified antigen-binding fragment of Claim 14 wherein said antibody or antigen-binding fragment is characterized by increased *in vivo* serum half-life, compared to the unmodified antibody or antigen-binding fragment.
16. The modified antibody or modified antigen-binding fragment of Claim 15 wherein said antibody or antigen-binding fragment binds GPIIb/IIIa and/or  $\alpha_v\beta_3$  with substantially the same affinity as the unmodified antibody or antigen-binding fragment.
- 10 17. The modified antibody or modified antigen-binding fragment of Claim 16 wherein said antibody or antigen-binding fragment is characterized by decreased immunogenicity, compared to the unmodified antibody.
- 15 18. The modified antibody or modified antigen-binding fragment of Claim 16 wherein said antibody or fragment is selected from the group consisting of 7E3, a humanized 7E3, and antigen-binding fragments thereof.
19. The modified antibody or modified antigen-binding fragment of Claim 18 wherein said humanized 7E3 or antigen-binding fragment thereof is a chimeric 7E3 antibody or chimeric antigen-binding fragment thereof.
- 20 20. The modified antibody or modified antigen-binding fragment of Claim 16 wherein said organic moiety is a hydrophilic polymeric group.

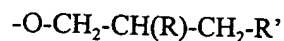
21. The modified antibody or modified antigen-binding fragment of Claim 20 wherein said hydrophilic polymeric group is a linear or branched polyalkane glycol chain, a carbohydrate chain, an amino acid chain or a polyvinyl pyrrolidone chain, and wherein said hydrophilic polymeric group has a molecular weight of about 800 to about 120,000 Daltons.
22. The modified antibody or modified antigen-binding fragment of Claim 21 wherein said hydrophobic polymeric group is a linear or branched polyalkane glycol chain with a molecular weight greater than 2,000 Daltons.
23. The modified antibody or modified antigen-binding fragment of Claim 20 wherein said hydrophilic polymeric group is a linear or branched polyethylene glycol chain or a linear or branched substituted polyethylene glycol chain that is substituted with an alkyl group, a C<sub>6</sub>-C<sub>40</sub> fatty acid group, a C<sub>6</sub>-C<sub>40</sub> fatty acid ester group, a lipid group or a phospholipid group.
24. The modified antibody or modified antigen-binding fragment of Claim 23 wherein said hydrophilic polymeric group is a linear or branched polyethylene glycol chain that is terminally substituted with an alkyl group, a C<sub>6</sub>-C<sub>40</sub> fatty acid group, a C<sub>6</sub>-C<sub>40</sub> fatty acid ester group, a lipid group or a phospholipid group.
25. The modified antibody or modified antigen-binding fragment of Claim 24 wherein said substituent is palmitoyl.
26. The modified antibody or modified antigen-binding fragment of Claim 23 wherein said antibody or fragment is selected from the group consisting of 7E3, a humanized 7E3, and antigen-binding fragments thereof.
27. The modified antibody or modified antigen-binding fragment of Claim 23 wherein an organic moiety is covalently bonded to at least one carboxyl-terminus of said antibody or antigen-binding fragment.

28. The modified antigen-binding fragment of Claim 27 wherein said antigen-binding fragment is an Fab.
29. The modified antigen-binding fragment of Claim 28 wherein said Fab is an achromopeptidase fragment.
- 5 30. The modified antibody or modified antigen-binding fragment of Claim 27 wherein said carboxyl-terminus is the carboxyl-terminus of the heavy chain.
31. The modified antibody or modified antigen-binding fragment of Claim 30 further comprising one to about four additional organic moieties which are each independently covalently bonded to the side chain sulfur atom of a  
10 cysteinyl residue.
32. The modified antibody or modified antigen-binding fragment of Claim 31 wherein the heavy chain comprises said cysteinyl residue.
33. The modified antibody or modified antigen-binding fragment of Claim 26 wherein an organic moiety is covalently bonded to the side chain sulfur atom  
15 of at least one cysteinyl residue.
34. The modified antibody or modified antigen-binding fragment of Claim 33 wherein the heavy chain comprises said cysteinyl residue.
35. The modified antigen-binding fragment of Claim 34 wherein said antigen binding fragment is a Fab' and the organic moiety is bonded to a sulfur atom  
20 of at least one heavy chain cysteinyl residue that forms an inter-chain disulfide bond.
36. The modified antibody or modified antigen-binding fragment of Claim 16 wherein said organic moiety is a linear or branched C<sub>6</sub>-C<sub>40</sub> fatty acid.

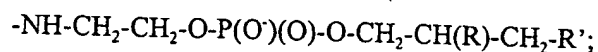
37. The modified antibody or modified antigen-binding fragment of Claim 36 wherein said antibody or fragment is selected from the group consisting of 7E3, a humanized 7E3, and antigen-binding fragments thereof.
- 5 38. The modified antibody or modified antigen-binding fragment of Claim 37 wherein an organic moiety is covalently bonded to at least one carboxyl-terminus of said antibody or antigen-binding fragment.
39. The modified antigen-binding fragment of Claim 38 wherein said antigen-binding fragment is an Fab.
- 10 40. The modified antibody or modified antigen-binding fragment of Claim 38 further comprising one to about four additional organic moieties which are each independently covalently bonded to the side chain sulfur atom of a cysteinyl residue.
- 15 41. The modified antibody or modified antigen-binding fragment of Claim 37 wherein an organic moiety is covalently bonded to the side chain sulfur atom of at least one cysteinyl residue.
42. The modified antigen-binding fragment of Claim 41 wherein said antigen binding fragment is an Fab' and the organic moiety is bonded to a sulfur atom of at least one heavy chain cysteinyl residue that forms an inter-chain disulfide bond.
- 20 43. The modified antibody or modified antigen-binding fragment of Claim 16 wherein said organic moiety is a linear or branched C<sub>6</sub>-C<sub>40</sub> fatty acid ester.
44. The modified antibody or modified antigen-binding fragment of Claim 43 wherein said antibody or fragment is selected from the group consisting of 7E3, a humanized 7E3, and antigen-binding fragments thereof.

45. The modified antibody or modified antigen-binding fragment of Claim 44 wherein an organic moiety is covalently bonded to at least one carboxyl-terminus of said antibody or antigen-binding fragment.
- 5 46. The modified antigen-binding fragment of Claim 45 wherein said antigen-binding fragment is an Fab.
47. The modified antibody or modified antigen-binding fragment of Claim 45 further comprising one to about four additional organic moieties which are each independently covalently bonded to the side chain sulfur atom of a  
10 cysteinyl residue.
48. The modified antibody or modified antigen-binding fragment of Claim 44 wherein an organic moiety is covalently bonded to the side chain sulfur atom of at least one cysteinyl residue.
49. The modified antigen-binding fragment of Claim 48 wherein said antigen  
15 binding fragment is a Fab' and the organic moiety is bonded to a sulfur atom of at least one heavy chain cysteinyl residue that forms an inter-chain disulfide bond.
50. The modified antibody or modified antigen-binding fragment of Claim 16 wherein said organic moiety is a lipid or phospholipid.

51. The modified antibody or modified antigen-binding fragment of Claim 50 wherein said organic moiety is represented by a Structural Formula selected from the group consisting of:



5 and



10 wherein R and R' are each, independently, -OH or a group represented by -X-Y, wherein -X- is -O-, -O-C(O)-, -S-, -O-S(O)<sub>2</sub>-O-, -NH-C(O)- or -O-CH=CH-, and Y is a saturated or unsaturated C<sub>6</sub> to about C<sub>40</sub> hydrocarbon group, and wherein both R and R' are not -OH.

52. The modified antibody or modified antigen-binding fragment of Claim 51 wherein said antibody or fragment is selected from the group consisting of 7E3, a humanized 7E3, and antigen-binding fragments thereof.

53. The modified antibody or modified antigen-binding fragment of Claim 52 wherein an organic moiety is covalently bonded to at least one carboxyl-terminus of said antibody or antigen-binding fragment.

54. The modified antigen-binding fragment of Claim 54 wherein said antigen-binding fragment is an Fab.

55. The modified antibody or modified antigen-binding fragment of Claim 53 further comprising one to about four additional organic moieties which are each independently covalently bonded to the side chain sulfur atom of a cysteinyl residue.



56. The modified antibody or modified antigen-binding fragment of Claim 52 wherein an organic moiety is covalently bonded to the side chain sulfur atom of at least one cysteinyl residue.
57. The modified antigen-binding fragment of Claim 56 wherein said antigen binding fragment is a Fab' and the organic moiety is bonded to a sulfur atom of at least one heavy chain cysteinyl residue that forms an inter-chain disulfide bond.
58. A method of inhibiting thrombosis in a patient comprising administering to said patient an effective amount of a modified antibody or modified antigen-binding fragment of Claim 12.
59. The method of Claim 58 wherein said modified antibody or modified antigen-binding fragment is characterized by increased *in vivo* half-life compared to the unmodified antibody or antigen-binding fragment.
60. The method of Claim 59 wherein said modified antibody or modified antigen-binding fragment further specifically binds  $\alpha_v\beta_3$ .
61. The method of Claim 60 wherein said modified antibody or modified antigen-binding fragment is characterized by decreased immunogenicity compared to the unmodified antibody or antigen-binding fragment.
62. The method of Claim 60 wherein said antibody or fragment is selected from the group consisting of 7E3, a humanized 7E3, and antigen-binding fragments thereof.
63. A method of inhibiting stenosis and/or restenosis following a vascular intervention procedure in a human comprising administering to said human an effective amount of a modified antibody or modified antigen-binding fragment of Claim 12.

64. The method of Claim 63 wherein said modified antibody or modified antigen-binding fragment is characterized by increased *in vivo* half-life compared to the unmodified antibody or antigen-binding fragment.
- 5 65. The method of Claim 64 wherein said modified antibody or modified antigen-binding fragment further specifically binds  $\alpha_v\beta_3$ .
66. The method of Claim 65 wherein said modified antibody or modified antigen-binding fragment is characterized by decreased immunogenicity compared to the unmodified antibody or antigen-binding fragment.
- 10 67. The method of Claim 65 wherein said antibody or fragment is selected from the group consisting of 7E3, a humanized 7E3, and antigen-binding fragments thereof.
- 15 68. A method of preventing ischemia in a human comprising administering to said human an effective amount of a modified antibody or modified antigen-binding fragment of Claim 12.
69. The method of Claim 68 wherein said modified antibody or modified antigen-binding fragment is characterized by increased *in vivo* half-life compared to the unmodified antibody or antigen-binding fragment.
- 20 70. The method of Claim 69 wherein said modified antibody or modified antigen-binding fragment further specifically binds  $\alpha_v\beta_3$ .
71. The method of Claim 70 wherein said modified antibody or modified antigen-binding fragment is characterized by decreased immunogenicity compared to the unmodified antibody or antigen-binding fragment.

72. The method of Claim 70 wherein said antibody or fragment is selected from the group consisting of 7E3, a humanized 7E3, and antigen-binding fragments thereof.
- 5 73. A method of inhibiting the growth and/or metastasis of a tumor in a human comprising administering to said human an effective amount of a modified antibody or modified antigen-binding fragment of Claim 12.
74. The method of Claim 73 wherein said modified antibody or modified antigen-binding fragment is characterized by increased *in vivo* half-life compared to the unmodified antibody or antigen-binding fragment.
- 10 75. The method of Claim 74 wherein said modified antibody or modified antigen-binding fragment further specifically binds  $\alpha_v\beta_3$ .
76. The method of Claim 75 wherein said modified antibody or modified antigen-binding fragment is characterized by decreased immunogenicity compared to the unmodified antibody or antigen-binding fragment.
- 15 77. The method of Claim 75 wherein said antibody or fragment is selected from the group consisting of 7E3, a humanized 7E3, and antigen-binding fragments thereof.
78. The method of Claim 75 wherein said modified antibody inhibits  $\alpha_v\beta_3$ -mediated angiogenesis and/or platelet-mediated metastasis.
- 20 79. A method of inhibiting a process mediated by the binding of a ligand to GPIIb/IIIa,  $\alpha_v\beta_3$  and/or Mac-I expressed on the plasma membrane of a cell in a human, comprising administering to said human an effective amount of modified antibody or antigen-binding fragment of Claim 2.

80. A method of inhibiting angiogenesis in a human comprising administering to said human an effective amount of a modified antibody or a modified antigen-binding fragment that specifically binds  $\alpha_v\beta_3$ , the modification comprising one or more organic moieties which are covalently bonded to said antibody or antigen-binding fragment.
81. The method of Claim 80 wherein said modified antibody or modified antigen-binding fragment is characterized by increased *in vivo* half-life compared to the unmodified antibody or antigen-binding fragment.
82. The method of Claim 81 wherein said modified antibody or modified antigen-binding fragment further specifically binds GPIIb/IIIa.
83. The method of Claim 82 wherein said modified antibody or modified antigen-binding fragment is characterized by decreased immunogenicity compared to the unmodified antibody or antigen-binding fragment.
84. The method of Claim 83 wherein said antibody or fragment is selected from the group consisting of 7E3, a humanized 7E3, and antigen-binding fragments thereof.

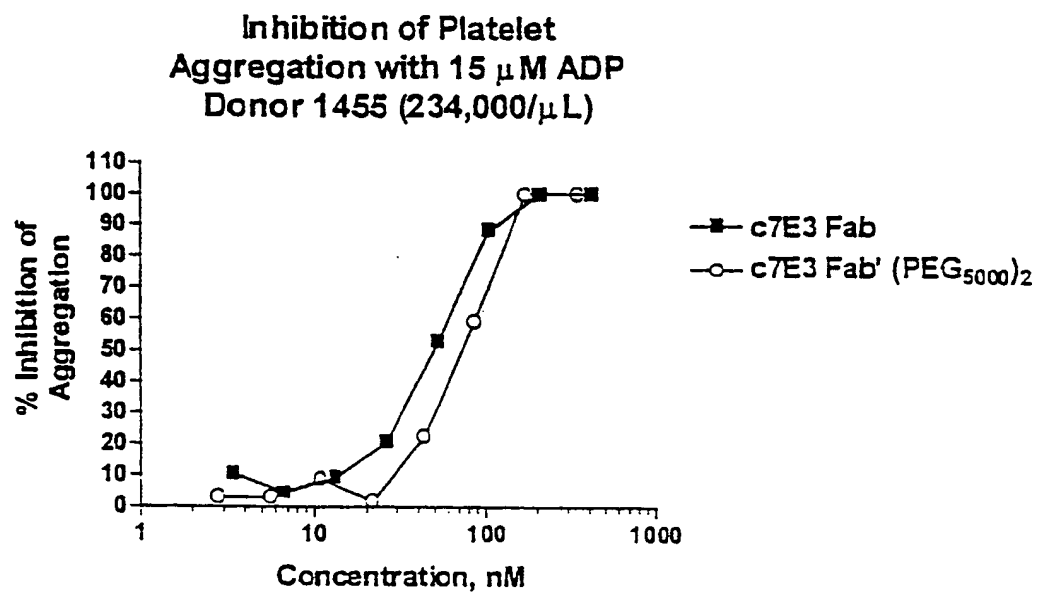


Figure 1

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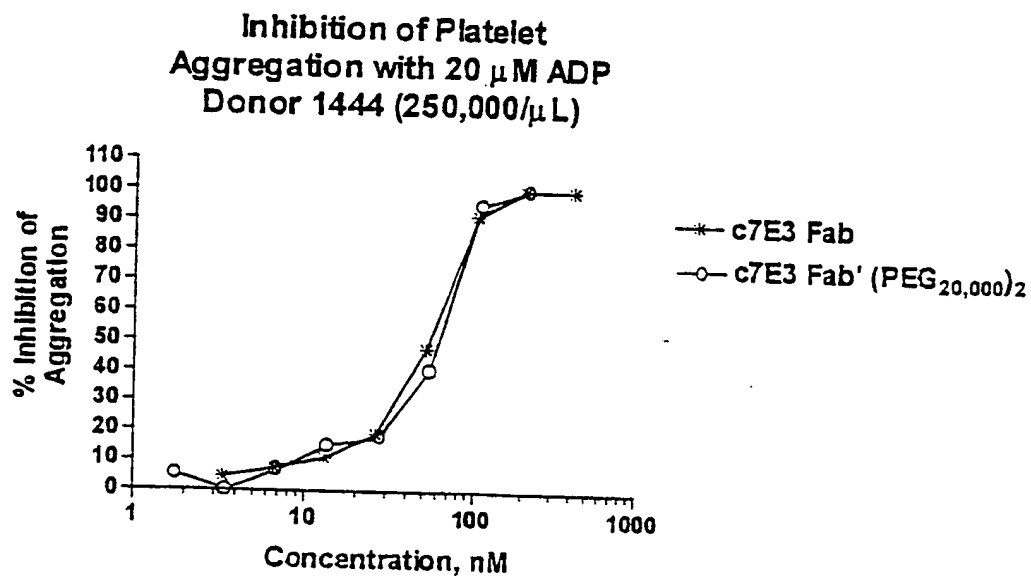


Figure 2

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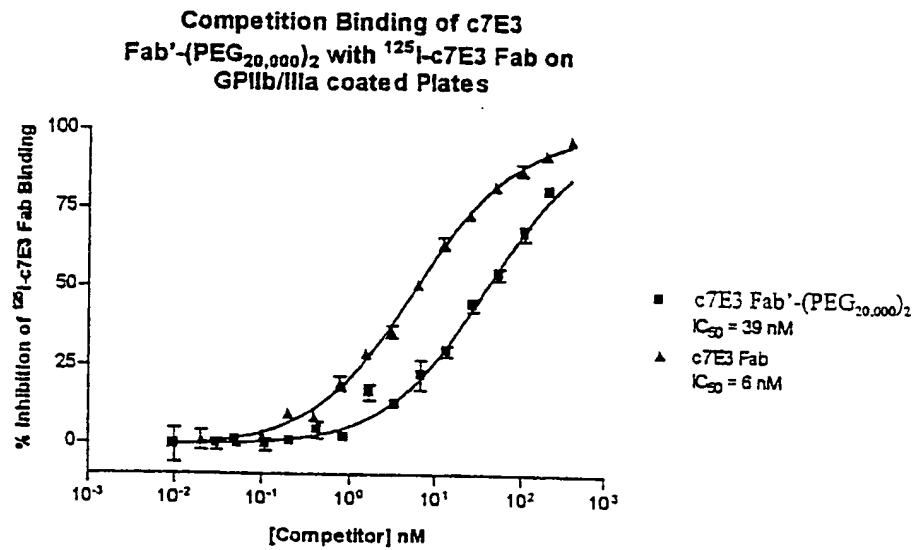


Figure 3

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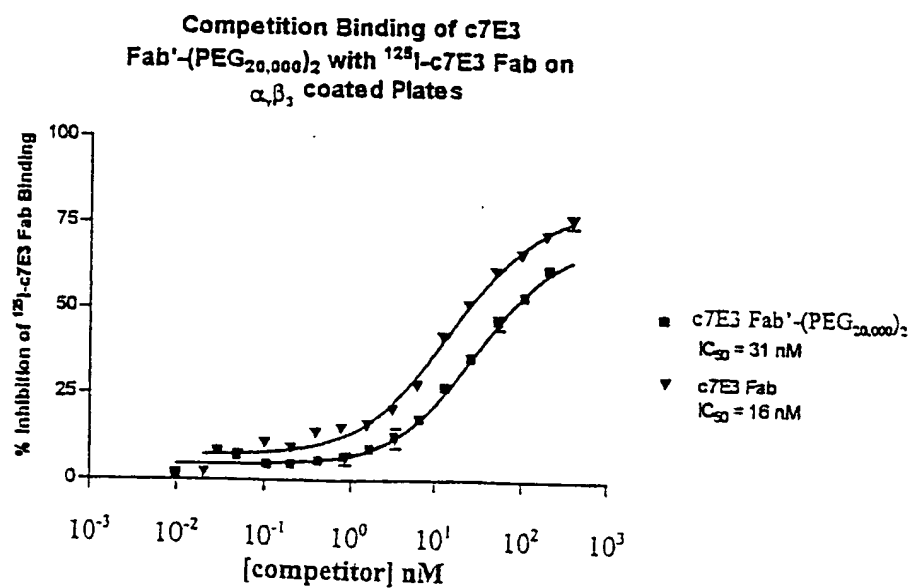


Figure 4



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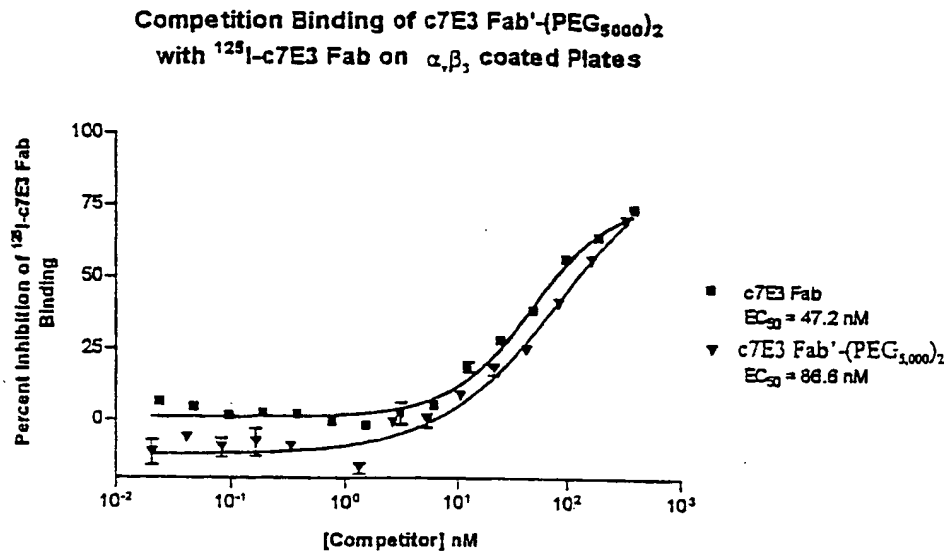


Figure 5

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Competition Binding of c7E3 Fab'-(PEG<sub>5,000</sub>)<sub>2</sub>  
with <sup>125</sup>I-c7E3 Fab on GPIIb/IIIa coated Plates

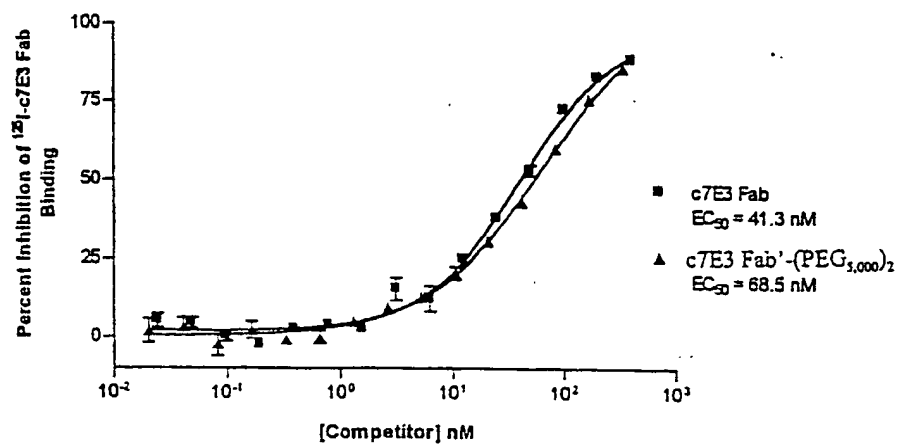


Figure 6.

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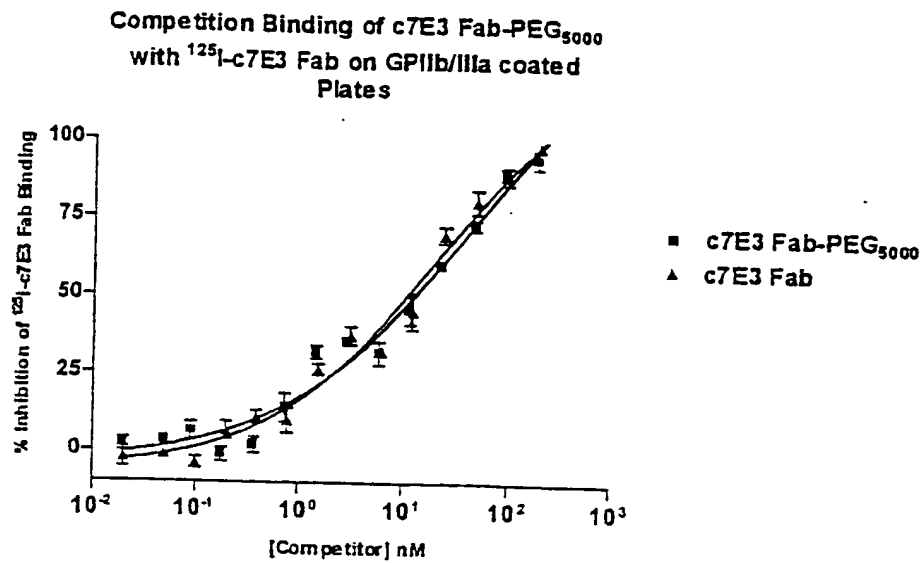


Figure 7

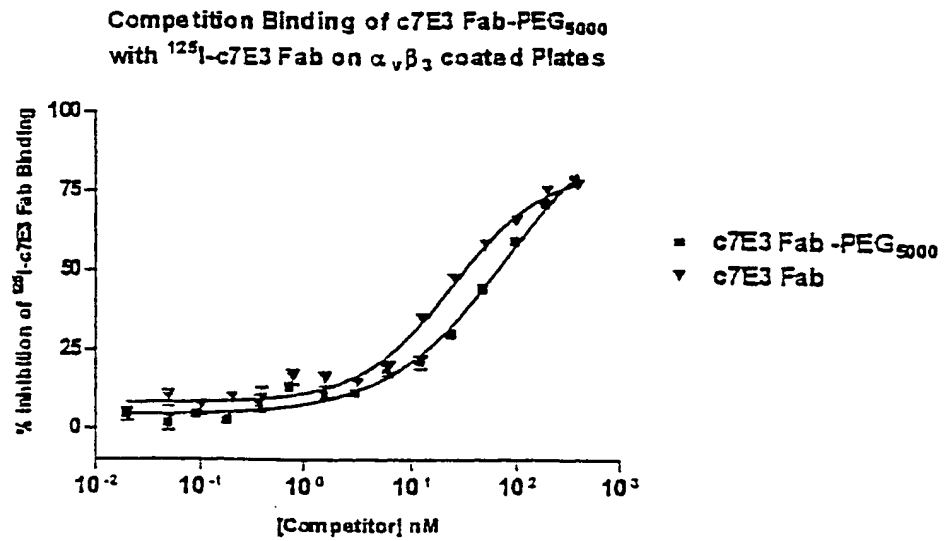


Figure 8

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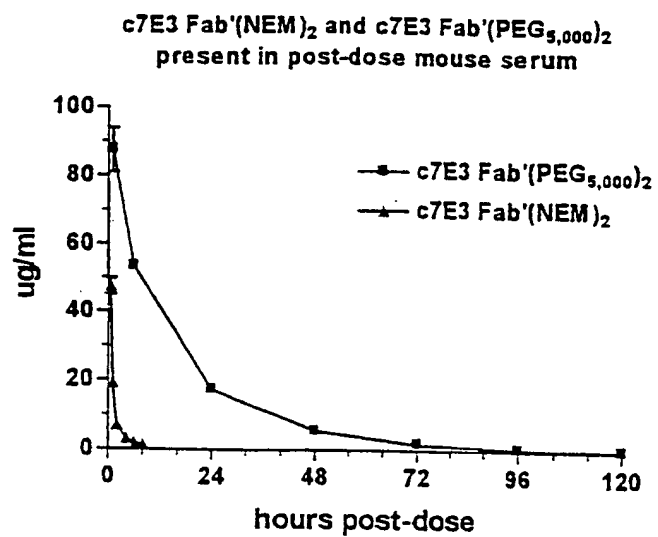


Figure 9

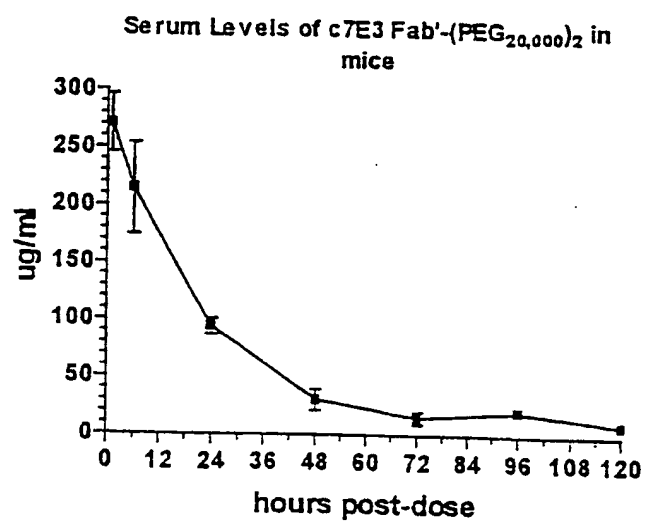


Figure 10

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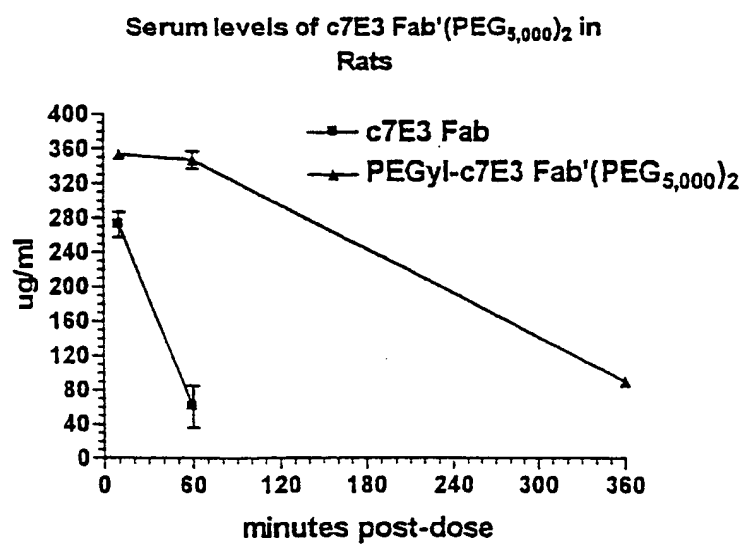


Figure 11

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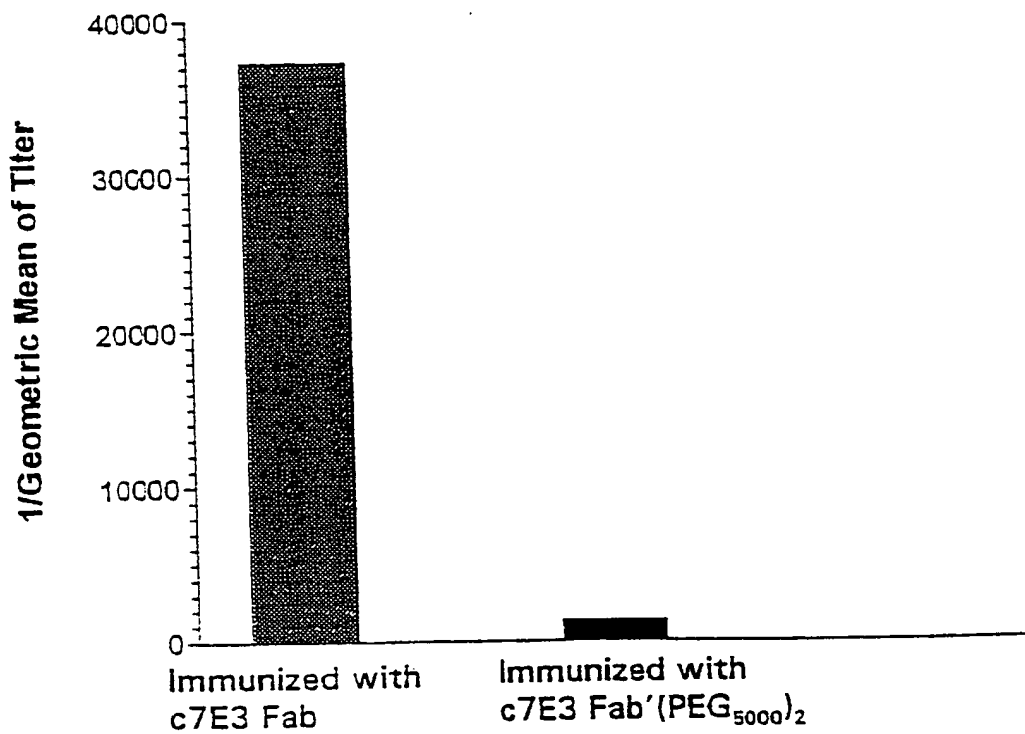


Figure 12



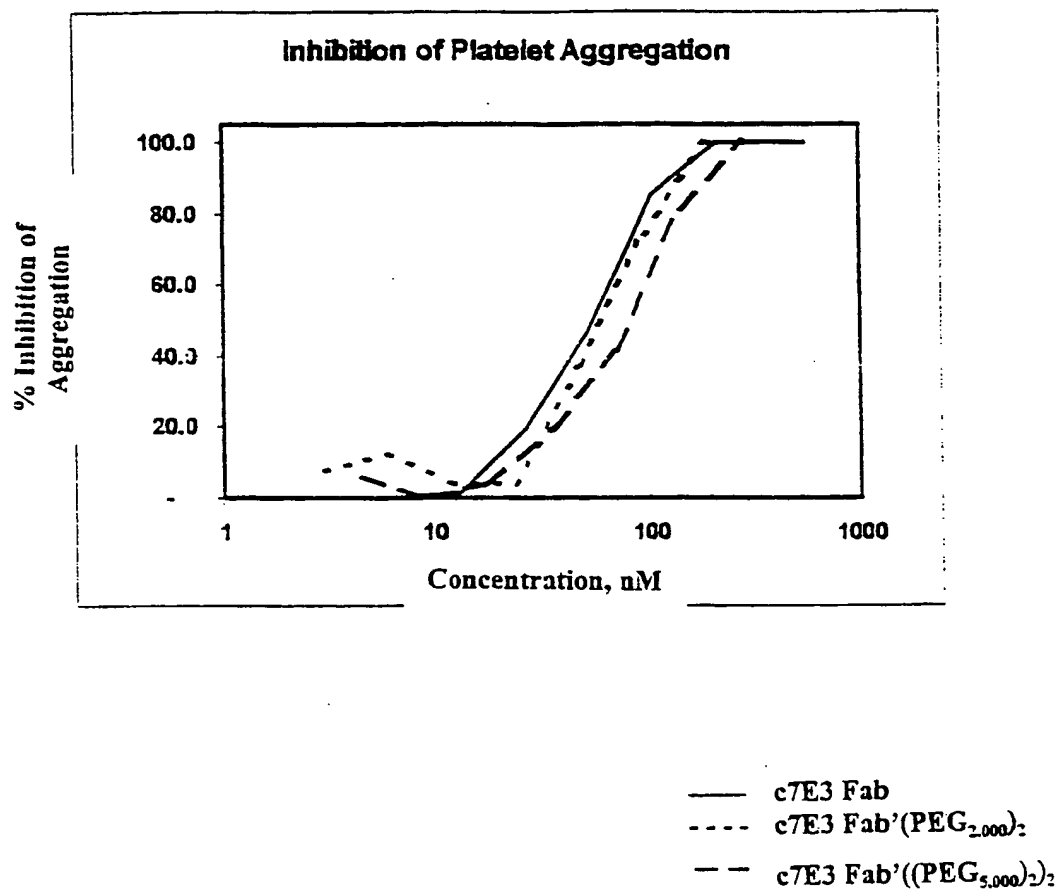


Figure 13

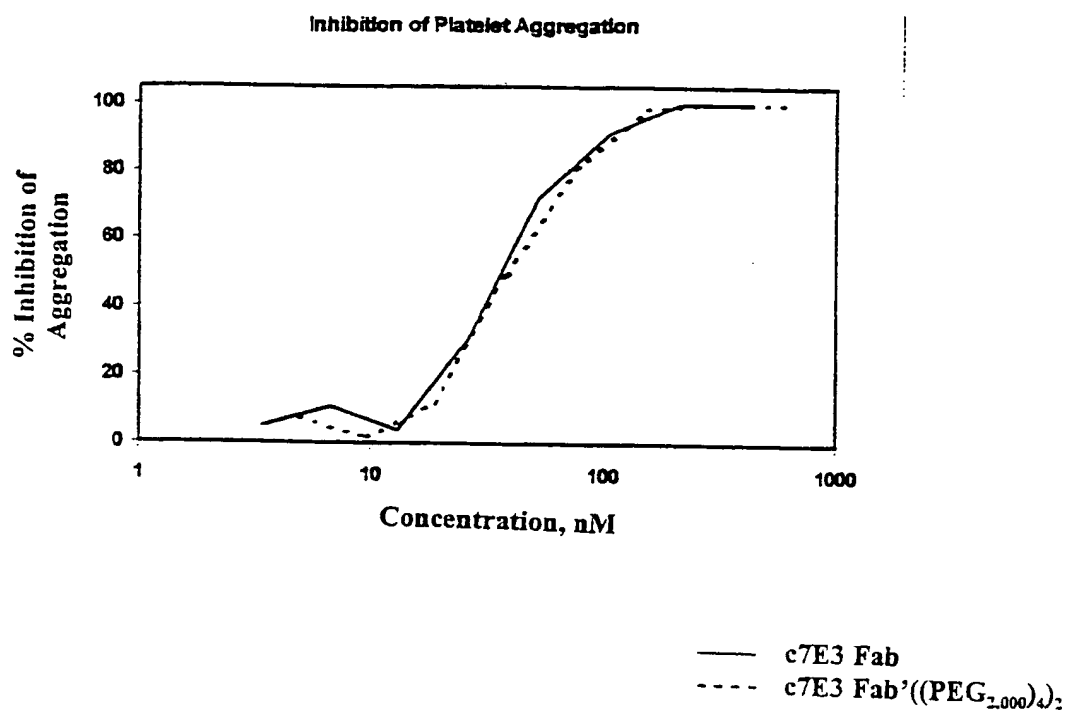


Figure 14

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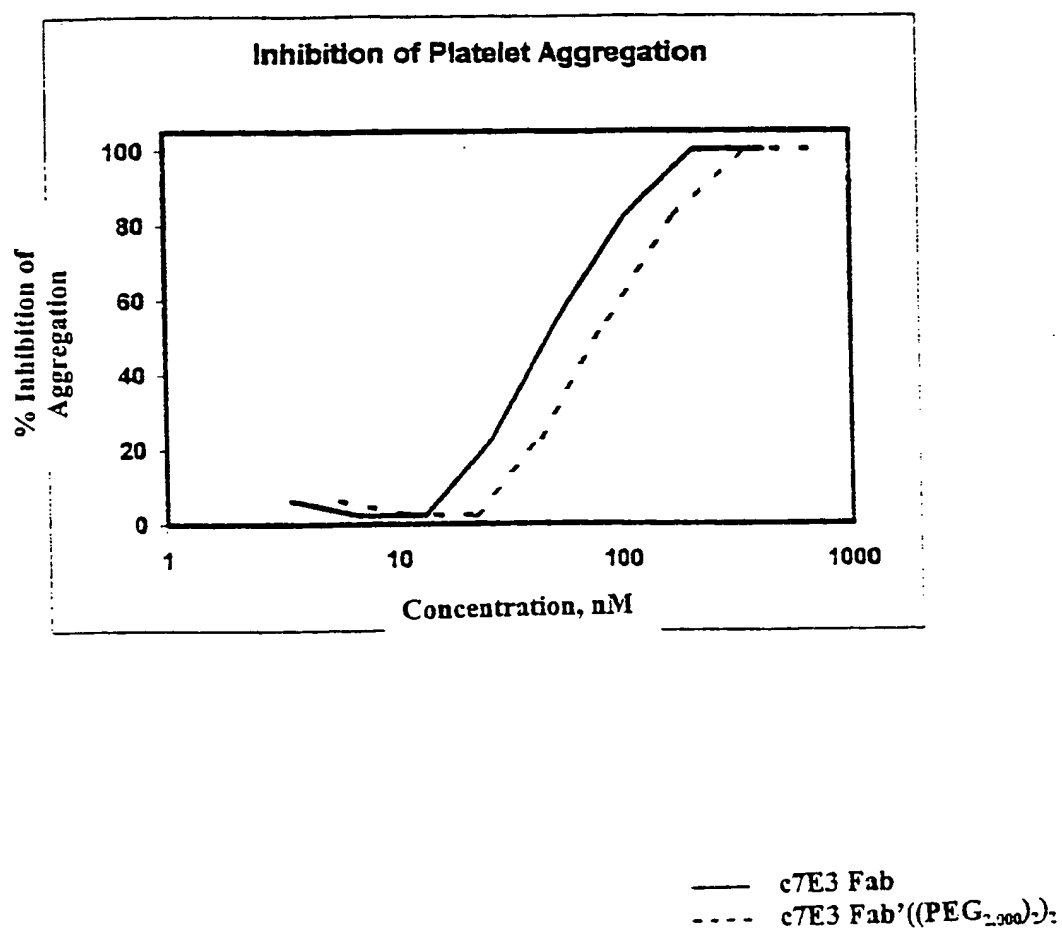


Figure 15